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THE STRUCTURE AND MANNER OF DIVISION OF THE NUCLEI IN THE VEGETATIVE MYCELIUM OF GELASINOSPORA TETRASPERMA DOWD.[1]

A. BAKERSPIGEL

Abstract

The nuclei in the vegetative mycelium of *Gelasinospora tetrasperma* do not divide in a manner directly comparable to ordinary mitosis. They divide without the aid of a spindle and individually recognizable chromosomes do not appear to align themselves on a metaphase plate. It is suggested that the dividing central body may function as a mechanical device to separate the divided chromatin and that the densely stained granule present in the chromatin of these nuclei is important to the division of both the chromatin and the central body.

Introduction

In two recent reports (4, 5) the writer described the structure and manner of division of the nuclei in the vegetative mycelium of the ascomycete, *Neurospora crassa*. He noted that *N. crassa* was another example of a fungus in which vegetative nuclei do not divide in a manner directly comparable to ordinary mitosis. Instead complexes of chromosomal filaments contract, then constrict at the mid-region, and finally separate into two sister nuclei. At the same time the central body also constricts so that at the end of division each sister nucleus contains presumably equal portions of the original chromatin and central body.

Several species of another ascomycete, *Gelasinospora*, have become the subject of cytogenetic and taxonomic studies (1, 6, 7, 8, 9, 10, 11). However, with the exception of the earlier report by Dowding and Bakerspigel (7) and the more recent one by Dowding (9) on the migrating nuclei in the hyphae of *Gelasinospora tetrasperma*, no additional observations have been recorded on the vegetative nuclei in any species of this fungus. Since chromosomal counts have also been made in the developing asci of some of these species (11), it was of interest to determine how the vegetative nuclei in at least one species of *Gelasinospora* behaved during division. Furthermore, the close taxonomic relationship that exists between *Neurospora* and *Gelasinospora* afforded an opportunity of comparing the vegetative nuclei in these two ascomycetes.

[1]Manuscript received September 8, 1958.
Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario. Present address: Department of Bacteriology, The New Mount Sinai Hospital, Toronto, Ontario, Canada.

[The previous number of Can. J. Microbiol. (**5**, 1–124) was issued March 2, 1959.]

Materials and Methods

Cultures

Nuclei in the vegetative hyphae of three strains of *G. tetrasperma* were studied. These included a fast-fruiting heterokaryon (+ −) obtained from Dr. E. S. Dowding, University of Alberta, as well as plus (+) and minus (−) homokaryons isolated from this heterokaryon. The methods employed in the isolation of these homokaryons and in determining their mating behavior have been described in detail elsewhere (7).

Cytological Preparations

Heterokaryotic mycelium. — Normal-sized ascospores were inoculated directly on sterile strips of cellophane placed on the surface of malt–agar medium. When germ tubes or hyphae of desired length had been produced, the cultures were fixed *in situ* with acetic acid – alcohol (1:3) or osmium tetroxide vapors (4, 5).

Homokaryotic mycelium.—Suspensions in sterile distilled water or hyphal fragments removed from growing cultures of (+) and (−) homokaryons were pipetted over cellophane strips placed on malt – agar medium. When actively growing hyphae of desired length were obtained the cultures were fixed *in situ* as described above.

Staining.—Acetic acid – alcohol-fixed cultures were stained by the HCl–Giemsa and Feulgen techniques. After osmium tetroxide fixation hyphae were stained with iron alum haematoxylin. All of these techniques have been described in detail by the writer (2, 3, 4).

Nuclei in living mycelium.—Slide cultures of living hyphae for study with the phase-contrast microscope were also prepared as outlined by the writer (2, 3, 4).

Observations

Since no apparent differences were noted in the structure and manner of division of nuclei in the vegetative hyphae of the heterokaryotic or homokaryotic strains studied, only one description will be given in this report.

FIGS. 1–16. HCl–Giemsa.

FIG. 1. An interdivisional (resting) nucleus situated at the septum of a cell. Magnification, 4860.

FIGS. 2–7. Migrating nuclei of various shapes. In Figs. 2 and 3 the nuclei were fixed while passing through septal pores. Magnification of Figs. 3 and 4, 4860; Figs. 5–7, 3670.

FIGS. 8–16. Dividing nuclei. Magnification, 3670.

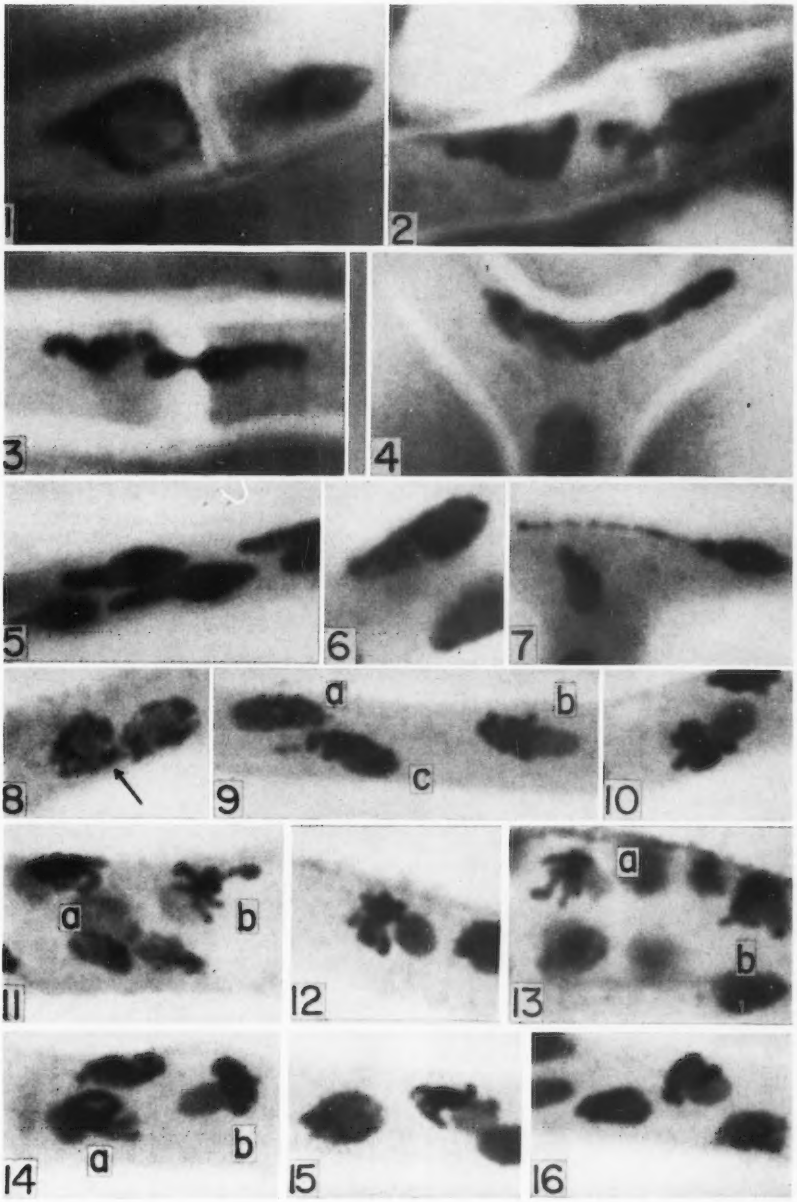
FIG. 8. Two nuclei at beginning of division. Arrow points to the densely stained granule in the chromatin of this nucleus.

FIG. 9. Division continuing. (*a*) The coarsely granular chromatin shown contracting and withdrawing from the central body. (*b*) The chromatin has now become a complex of chromosomal filaments. (*c*) A later divisional stage comparable to that in Fig. 16.

FIGS. 10–14. As division proceeds, variously arranged complexes of chromosomal filaments are formed. These are evident in Figs. 11, *a* and *b*; 12, 13, *a* and 14, *b*. Note also the densely stained granule in 13, *b*.

FIG. 15. The chromosomes now begin to contract into a more compact unit.

FIG. 16. The chromosomes have now become a solid, densely stained mass of chromatin. Similar stages are seen in Figs. 13, *b* and 14, *b*.



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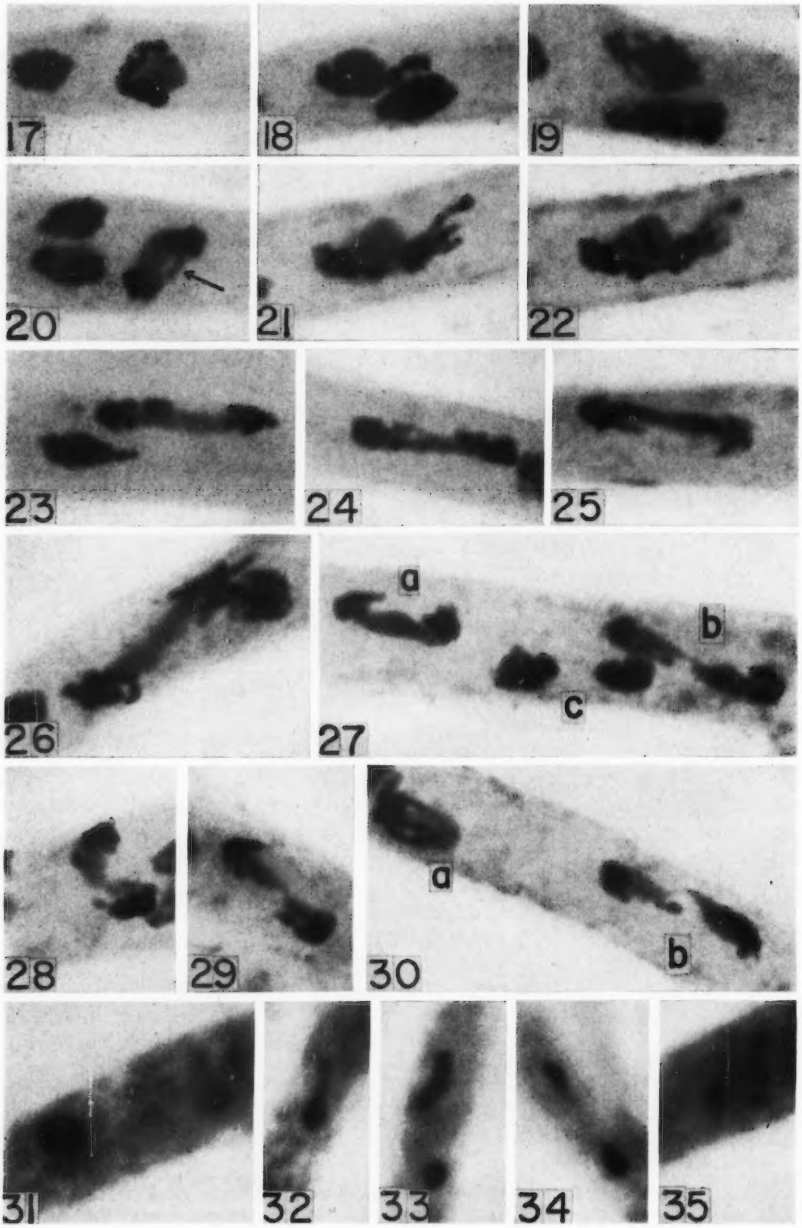

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References to individual photomicrographs will not be given in the text and the reader is referred to the legends for their interpretation.

Interdivisional Nuclei (Figs. 1–7, 31, 36–40)

Stained preparations reveal that interdivisional (resting) nuclei in young germ tubes or older hyphae are composed of finely granular or beaded chromatin, which wholly or partially envelops spherical or oval central bodies. When these nuclei are at rest they may be spherical or lens-shaped. However, when they migrate from one cell to another they may become elongated or serpentine in shape. Nuclei which migrate from cells in older portions of a hypha toward actively growing tips may be "sperm-shaped". Such nuclei usually have "tails" of beaded chromatin of various lengths which terminate in a densely stained granule. Feulgen preparations of interdivisional nuclei are similar to those stained by the HCl–Giemsa technique with the usual exception that the Feulgen-negative central body is not evident.

Iron alum haematoxylin preparations of resting nuclei show them to be composed of the familiar, densely stained, central bodies surrounded by variously shaped, unstained or faintly stained areas. A nuclear membrane is not visible. Granular and filamentous mitochondria are present in the hyphae, the filamentous form being found more frequently near hyphal tips and in older vacuolated cells.

Dividing Nuclei (Figs. 8–16, 17–30, 32–35, 41–46)

Nuclear division, as observed in the HCl–Giemsa preparations, begins with the granular chromatin becoming coarser, contracting, and staining more intensely. As the chromatin contracts still further it becomes withdrawn from the central body to which it may at this stage still remain attached at one side. The chromatin then becomes reorganized forming a complex of

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FIGS. 17–30. HCl–Giemsa. Magnification, 3670.

FIGS. 17, 18. Division continuing. The coarsely granular chromatin which forms an irregular bar is shown beginning to stretch around the central body.

FIG. 19. The central body at this stage may also begin to elongate. In this nucleus it is also seen constricting.

FIG. 20. The bar of chromatin has divided into two portions each of which is now situated at opposite sides of the elongating central body. The arrow points to the two granules situated on the strand connecting the two separated portions of chromatin.

FIGS. 21, 22. The same nucleus at two focal levels. The portions of the dividing chromatin have not fully contracted prior to their separation.

FIGS. 23–27, *a*. Division drawing to a close. The central bodies have elongated still further and are shown becoming progressively more constricted.

FIGS. 27, *b*–29. End of division. The constricted nuclei have nearly separated. In Fig. 27, *b* the two special granules have separated and are seen attached to each portion of the divided chromatin.

FIG. 30. Postdivision. Two separated sister nuclei shown at *b*. Similar figures shown in Fig. 27, *c*. The divisional stage illustrated at *a* is comparable to those shown in Figs. 14, *a* and 15.

FIGS. 31–35. Iron alum haematoxylin. Magnification, 3670.

FIG. 31. Interdivisional nuclei.

FIGS. 32, 33. Division proceeding. The central bodies shown elongated. The one in Fig. 32 is beginning to constrict.

FIG. 34. End of division. The two portions of the central body have just separated.

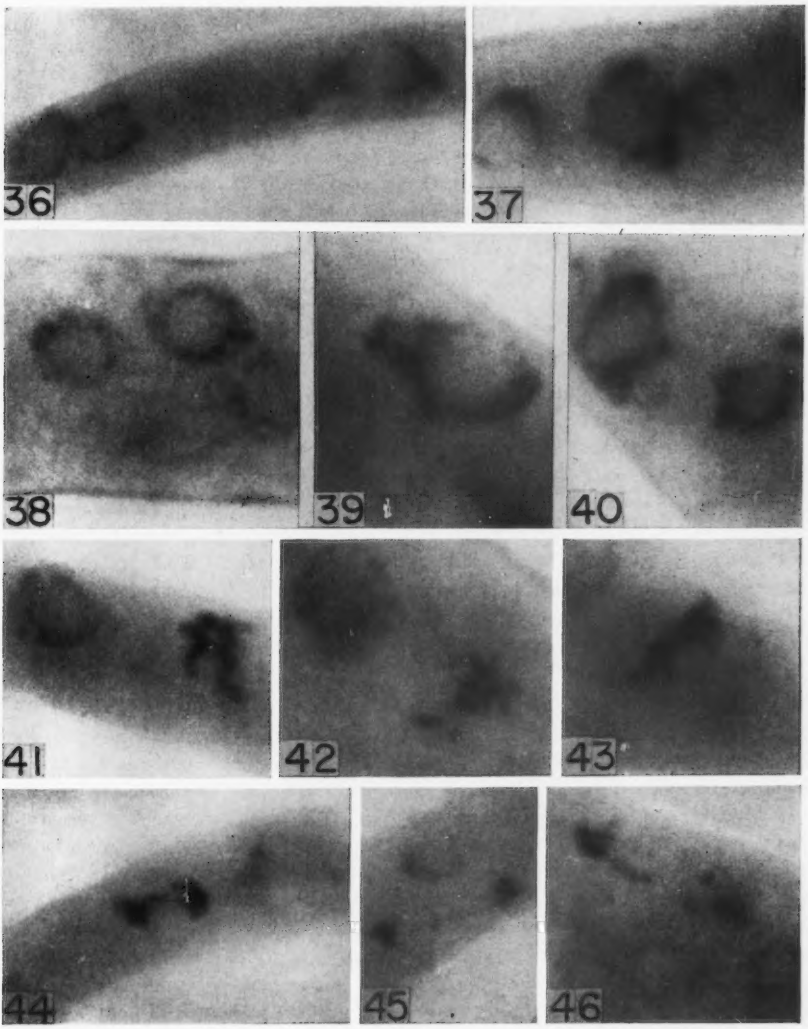
FIG. 35. Postdivision. Two maturing sister nuclei lying side by side.

heavily stained chromosomal filaments which may be found situated at one side of the central body. Accurate counts of individual chromosomes are difficult to make; however, an estimate of the number of chromosomes within one of these complexes is a minimum of three and a maximum of four.

As division continues the chromosomal complex becomes more entangled forming a thick, irregularly shaped, and coarsely granular bar. This bar elongates, one end stretching across and around one side of the central body. Elongation of the chromatin continues till one portion (half?) of the bar has become separated from the other. This results in each of the two portions of chromatin lying on opposite sides of the central body, which in the meantime has also begun to elongate. In some instances the extremities of the separating portions of chromatin may still have a filamentous appearance. At this stage of division a narrow strand of chromatin may also be seen connecting the separated portions of chromatin. On this connecting strand are situated two granules, which are probably the products of division of the densely stained granule usually observed in an interdivisional nucleus (e.g. Figs. 8, 20.) The connecting strand then breaks apart and the elongated central body constricts at its mid-region. Finally, both ends of the dividing nucleus separate forming two sister nuclei each composed of presumably equal portions of the original chromatin and central body. The densely stained granules, which had been situated on the connecting strand, are now attached to each of the chromatin portions of the sister nuclei. A similar sequence of events can be observed in Feulgen-stained preparations with the obvious exception that the division of the Feulgen-negative central body cannot be followed. Figures 40–46 illustrate such a sequence.

As described in *N. crassa* (5) the only useful information that can be derived from the iron-alum-haematoxylin-stained preparations is that one is able to follow the division of the Feulgen-negative central body. At the beginning of division a spherical or oval central body elongates becoming rod-like in shape. As division proceeds the elongated central body narrows (constricts) at the mid-region till the two densely stained ends separate to become the central bodies of the sister nuclei. Each of the sister nuclei in these preparations is now found to be composed of a small, densely stained, oval central body surrounded by an unstained or faintly stained area of varying shape.

Nuclear membranes have not been detected surrounding any of the nuclei described above. Neither have spindles or metaphase plates been observed during nuclear division. In this connection it may be noted that the function ascribed to the central body in *N. crassa* applies also to those in *G. tetrasperma*. That is to say that the elongating central body may act as a mechanical device to help push the separated portions of chromatin farther apart. The densely stained granule, which is seen doubled during division, may also have some importance in the division of the nucleus.



FIGS. 36–46. Feulgen. Magnification of Figs. 36–43 and Fig. 46, 4680; Figs. 44 and 45, 3670.

FIGS. 36–40. Interdivisional nuclei. The densely stained granule is present in the nuclei shown in Figs. 37 and 40. Compare Fig. 39 with Fig. 1.

FIGS. 41–43. Divisional figures. Compare with Figs. 11–15 and Figs. 21, 22.

FIGS. 44–46. End of division. The portions of chromatin are shown separating. Compare with Figs. 27–30.

Living Nuclei

Nuclear division in living vegetative hyphae of *G. tetrasperma* is similar to that already reported in *N. crassa* and no description will be given; the reader is referred to the descriptions in (4) and (5). The writer, nevertheless, takes this opportunity to re-emphasize that during division a difficulty similar to that reported in *N. crassa* was also encountered in this study, viz., the "disappearance" of the elongating central body into the cytoplasm so that its final constriction cannot be observed towards the end of division.

Discussion

The structure of the interdivisional nuclei in *G. tetrasperma* and their manner of division closely resemble that described in *N. crassa* These similarities undoubtedly lend additional weight to the evidence that *N. crassa* and *G. tetrasperma* are closely related taxonomically.

The present study also reveals additional information which, when compared to several statements in the literature concerning vegetative nuclei, deserve comment at this time. For example, in 1950 Alexopoulos and Sung (1), in a report on a study of a new species of *Gelasinospora* (*G. autosteira*), remarked that "a large number of what appeared to be nuclei were stained in each cell, but these structures were so small that it was impossible to identify them with certainty." This result, which may have been due primarily to their methods of fixation and staining, is a further example of the scanty observations recorded in the literature describing the structure and behavior of the vegetative fungal nuclei (4).

In an earlier report describing the migrating nucleus in the vegetative mycelium of *G. tetrasperma*, Dowding and Bakerspigel (7) recorded several methods and observations which in the light of present findings require some reinterpretation. In the first place, the methods employed by the authors in fixing and staining nuclei were, when compared to present techniques, inadequate with the result that what they assumed to be "nuclei" were most probably central bodies which had been stained directly with May–Grunewald or Giemsa. Secondly, because some of the cultures were fixed by air-drying, the stained preparations which resulted cannot be considered anything else but distorted facsimiles of nuclei stained during this study by more specific techniques. Among other examples that could be cited might be their method of preparing living cultures for phase-contrast microscopy (7). Indeed, critical examination of the living nuclei (in their Plate II, Fig. 34) reveals that the two dark bodies in the vacuolated hypha, which had been interpreted as contracted nuclei, probably represent a constricted central body in a dividing nucleus (7).

In the same publication the terms "expanded" and "contracted" were used by the authors to describe nuclei of various diameters while elongated, sinuous, "nonvacuolate", serpentine nuclei were termed "ophioplasts". The choice of the first two terms was unfortunate since in the writer's opinion these terms do not describe the *state* in which the nuclei are found but are

merely terms which describe the appearance of stained nuclei at various locations in a hypha. Thus, expanded nuclei may well be what the present writer has termed interdivisional nuclei while contracted nuclei may be the recent products of division or else sister nuclei at various stages of maturation. The nonvacuolate "ophioplasts" may be the poorly fixed and directly stained elongated central bodies of migrating nuclei. They may also be the elongated central bodies of dividing nuclei which, as has been shown in *N. crassa* and *G. tetrasperma*, divide by constriction quite rapidly.

The filaments which Dowding and Bakerspigel saw in the so-called "nuclear vacuoles" of stained and living preparations may have been in the first instance artifacts caused by poor fixation. In the second instance these filaments, which were again recently reported by Dowding (9), may have been filamentous mitochondria obstructing the continuous observation of a nucleus. Frequently mitochondria do pass over a nucleus in such a manner that they appear as radial filaments within the optically clear area that surrounds the central body. It is also not inconceivable that a vacuole may have been situated in a focal plane above or below the nucleus under examination. Since these vacuoles do contain short filaments and granules in motion, these inclusions may be misconstrued as filaments within the "nuclear vacuole". These conditions have been encountered by the writer during the study of living nuclei in vegetative hyphae of *N. crassa*, *G. tetrasperma*, and other fungi (4, 5).

G. tetrasperma is thus the second member of the ascomycetes in which the structure and behavior of vegetative nuclei during division has been described. Enough has now been observed in this fungus as well as in *N. crassa* and other fungi studied by the writer (2, 3, 4) to strengthen his opinion that further studies of this sort may well aid in clarifying certain problems prevalent not only in the cytology of the fungi but also in their taxonomy and relationship to other microorganisms.

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OBSERVATIONS ON THE AMINO ACID POOL AND CELLULAR LEAKAGE IN INTACT CELLS OF RHIZOBIUM MELILOTI[1]

D. C. JORDAN

Abstract

Cells of *Rhizobium meliloti* contained an intracellular pool of amino acids. The passage of C^{14}-histidine or C^{14}-glutamate into this pool apparently occurred against a concentration gradient and was energy dependent, being inhibited by azide and dinitrophenol. An internal bound form of C^{14}, containing a number of radioactive amino acids, was synthesized at the expense of radioactive pool components. This bound C^{14} consisted predominantly of several proteins whose syntheses were inhibited by chloramphenicol. Added histidine was not incorporated directly into this protein since the protein histidine was non-radioactive. When C^{14}-labelled cells were suspended in glucose-containing basal medium there was an efflux of pool amino acids into the extracellular environment. This leakage presumably was the result of simple diffusion since it was energy independent, occurred with the concentration gradient, and was appreciable at 5° C. Rapid leakage occurred at 30° C in the presence and absence of dinitrophenol for an initial period of 6 hours, after which time growth occurred in the absence of this inhibitor. During growth leakage ceased, and a considerable quantity of material passed back into the cells and was incorporated into a bound form. In the presence of dinitrophenol no growth occurred, and the outflow continued after the 6th hour, but at a much reduced rate. X irradiation increased the efflux of pool constituents, but the reason for this is conjectural.

Introduction

In previous work, when well-washed cells of *Rhizobium meliloti*, strain R_{21}, were ground with aluminum oxide, no free amino acids could be found in the resulting extract (14). Recent observations on sonic extracts of large numbers of cells have indicated to the author that an amino acid pool does exist in these Gram-negative organisms. This result is opposed to the findings of Taylor (18), who found amino acid pools in Gram-positive bacteria only, but is in agreement with the recent results of other authors including those of Cohen and Rickenberg (3) on *Escherichia coli*. The author has also observed that amino acids leak out into the extracellular fluid when rapidly washed cells of rhizobia are suspended in buffer. The present report concerns both the uptake of amino acids into the intracellular environment of *R. meliloti* and observations on the phenomenon of cellular leakage.

Methods

Cells of *Rhizobium meliloti*, strain R_{21}, were grown in 100 ml of $CaCO_3$-free yeast extract – mineral salts medium (19) at 28° C on a reciprocating shaker (100 cycles/minute, 2 in. stroke). After 24 hours the cells were removed, washed twice in 0.85% saline, and made up to a final volume of 1 ml with the same fluid. This suspension was then added to 8.5 ml of *carbohydrate-free* basal medium of the following composition: K_2HPO_4, 0.5 g; NH_4Cl, 0.53 g;

[1]Manuscript received October 30, 1958.
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph. Part of the program of the Legume Research Committee in Ontario.

$MgSO_4.7H_2O$, 0.2 g; $CaSO_4.2H_2O$, 0.2 g; NaCl, 0.1 g; $FeCl_3.6H_2O$, 0.1 g; and double-glass-distilled water, 1000 ml. After temperature equilibration for 15 minutes at 30° C, 0.5 ml of a solution of DL-histidine-α-C^{14} or DL-glutamate-α-C^{14} was added to make a final amino acid concentration of 1×10^{-4} *M*. During the incubation period 1-ml samples of the test mixture were withdrawn at various time intervals, centrifuged, and the supernatants discarded. The hot water procedure of Cohen and Rickenberg (3) was followed for the extraction of the intracellular pool, which was then analyzed for its C^{14} content. At the low concentration of amino acid employed, the error introduced by the presence of amino acid in the intercellular spaces of packed cells was negligible (3). The residue remaining after removal of the pool materials was washed twice in 3% formalin (12), once in double-distilled water, and the bound C^{14} determined. In some cases this material was extracted for 15 minutes at 90° C in 5% trichloroacetic acid, and then washed thoroughly in double-distilled water in order to remove nucleic acids. The loss of C^{14} as respiratory $C^{14}O_2$ was estimated by comparing the total radioactivity of a 1-ml sample of the incubation mixture at zero time with the total activity at the various time intervals. The decrease was assumed to be due to a loss of $C^{14}O_2$. In several instances the $C^{14}O_2$ produced was trapped as $BaC^{14}O_3$, and the radioactivity was measured at infinite thickness. The counting procedures were carried out with a shielded thin-window G.M. tube, the fluid samples being deposited, together with 3 drops of aqueous Tween 80, onto the surface of a lens paper disk held in an aluminum planchet. The background radiation amounted to an average of 10 counts per minute, and the results were reproducible to within 5%.

Since the resting cells of the test organism still maintained a very high endogenous respiration rate, the above procedures were repeated using cells which had been starved by shaking in distilled water for 20 hours at 28° C. Studies were also made on the effect of sodium azide, 2,4-dinitrophenol, and chloramphenicol on the accumulation of intracellular C^{14}.

The procedure of Gale (7) was used for the calculation of the intracellular "free space", assuming a specific volume of 0.72 for proteins. The "free space" originally contained within 1 mg of dried cells was calculated to be 4.1 μl.

Leakage of Intracellular Compounds

C^{14}-Labelled cells of the test organism were prepared by incubating washed cells for 2 to 3 hours at 30° C in basal medium containing 1 to 2 micromoles (0.5–1 μc) of histidine-α-C^{14}. After centrifugation the pellet of cells was rapidly washed twice in cold double-distilled water. The cells were then suspended in water, washed once, and placed in Seitz-filtered histidine-free basal medium containing 0.014 *M* glucose and, in some cases, 1×10^{-3} *M* dinitrophenol. Samples were obtained at different time intervals during incubation at 30° C or 5° C, the cells were removed by centrifugation, and the supernatants thus obtained were Millipore-filtered. Leakage C^{14} was determined by radioactive analyses of the cell-free filtrates. After hot

water treatment for pool extraction the cellular material was Millipore-filtered, and the filtrates were analyzed for pool C^{14}. The residues remaining on the filter disks were washed, dried, and examined *in situ* for the estimation of total bound C^{14}.

Viable counts, total cell counts, and nephelos determinations made throughout the course of similar experiments indicated that at 30° C no detectable growth or lysis of the cells occurred in the first 6 hours of incubation. After this time growth began in the absence, but not in the presence, of dinitrophenol. Upon incubation at 5° C a slight decrease in the total number of viable cells was noted.

In several instances, after incubation in radioactive histidine for the appropriate time interval, the cells were irradiated with a total dosage of 44,000 roentgens of X rays generated at 85 kv peak and 5 ma. This dose caused a 99.9% decrease in viable count without any observed cellular disruption. Leakage of C^{14}-compounds from a suspension of washed irradiated cells in glucose basal medium was then immediately compared with that found in a duplicate suspension of washed non-irradiated cells.

Analysis of C^{14}-Compounds

Pool amino acids, present both before and after incubation for 1 hour in histidine-α-C^{14} medium, were detected by two-dimensional paper chromatography in water-saturated phenol and in *sec*-butanol:88% aqueous formic acid:water (3:2:1). The identities of the materials which leaked from intact cells were established by the same technique.

Bound C^{14}, after extraction with hot trichloroacetic acid, was hydrolyzed in 6 *N* HCl for 20 hours at 121° C in a sealed pyrex tube. The resulting amino acids were extracted by the method of Block (2) and identified by chromatography. Radioactive areas on the various chromatograms were detected with a thin-window detector and by radioautography on no-screen X-ray film.

Sonic extracts from C^{14}-labelled and leached cells were prepared by a 10-minute treatment in a 10-kc sonic oscillator at a plate current of 1 amp, and, after centrifugation for 25 minutes at $3300 \times g$, various fractions were obtained by ammonium sulphate fractionation of the supernatants.

Results

In cells suspended in basal medium containing histidine-α-C^{14} the level of intracellular pool C^{14} rapidly increased up to a period of 1 hour after which time it decreased (Fig. 1). This decrease was coincident with increases in intracellular bound C^{14} and in the output of $C^{14}O_2$. By determining the concentrations of C^{14} in the extracellular fluid and in the internal "free space" it was calculated that at the end of 1 hour there was 243 times as much C^{14} internally as externally At this stage 83.8% of the recovered pool C^{14} was resident in histidine (Table I).

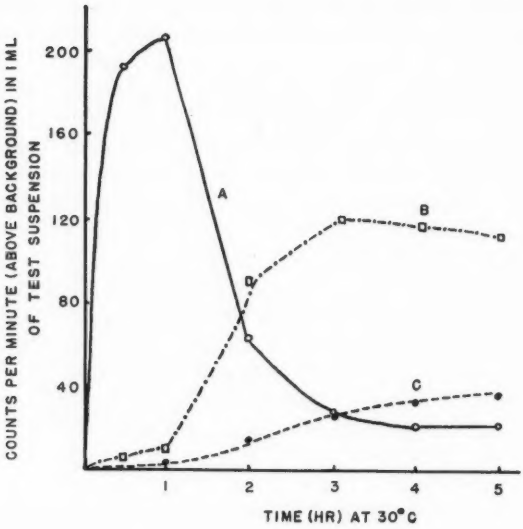

FIG. 1. Accumulation of C^{14} by non-growing cells of *R. meliloti* during incubation in the presence of DL-histidine-α-C^{14}. Curve A, intracellular pool C^{14}; curve B, intracellular bound C^{14}; curve C, respiratory $C^{14}O_2$.

TABLE I

Identification and radioactivity of amino acids detected in the intracellular pool and a protein hydrolyzate of C^{14}-labelled *R. meliloti*

Amino acid	Per cent of total recovered C^{14} activity	
	Pool (after 1 hr incubation in histidine-α-C^{14} medium)	Protein (hot trichloroacetic-acid-resistant) hydrolyzate
Alanine	1.4	15.4
α-Aminobutyric (?)	3.2	—
Aspartic	+	3.7
Glutamic	4.9	28.7
Glycine	+	2.9
Histidine	83.8	+
Leucine/isoleucine	+	+
Lysine/arginine	−	+
Proline	−	+
Serine	1.0	10.8
Threonine	−	20.8
Valine/methionine	2.3	8.3

NOTE: − = not detected; + = present but non-radioactive. The pool components also included (*a*) two unknown ninhydrin-positive compounds, one of which (R_f in phenol 0.28) contained 1% of the recovered C^{14}, and (*b*) five ninhydrin-negative fluorescent compounds, two of which together contained 2.2% of the recovered C^{14}. The protein hydrolyzate also contained two unknown ninhydrin-positive areas, one of which (R_f in phenol 0.27) contained 9.2% of the recovered radioactivity.

The presence of either 1.5×10^{-2} *M* sodium azide or 1×10^{-3} *M* dinitrophenol completely inhibited the passage of histidine into the cells. Chloramphenicol at a concentration of 50 μg/ml had little or no effect on the accumulation of pool C^{14}, but caused a 90% inhibition of the incorporation of C^{14} into the bound form. Starving the cells reduced their endogenous respiration and delayed the peak accumulation of bound C^{14} until the 4th hour.

Results similar to the above were obtained when glutamate-α-C^{14} replaced histidine-α-C^{14} in the basal medium, and in each case the total recovery of C^{14} as $C^{14}O_2$, pool, and bound forms amounted to more than 85%.

Although after 1 hour the greatest amount of pool C^{14} was present as histidine, this amino acid had undergone some degree of degradation, since $C^{14}O_2$ had been released and small amounts of radioactivity had appeared in glutamate and other compounds (Table I). The pool components found in rapidly washed cells prior to their addition to histidine or glutamate medium consisted of essentially the same compounds as shown in Table I, except that serine, valine and/or methionine, leucine and/or isoleucine, and one of the fluorescent areas were not detected. Since the amounts of the pool constituents were greatly reduced after washing it is possible that the above-mentioned compounds were present in undetectable amounts. The fluorescent areas appeared after phenol passage of the chromatograms and lost their fluorescence upon application of the second solvent. They have not as yet been identified.

Nucleic acid C^{14}, removed by the hot trichloroacetic acid extraction, varied between 25 and 28% of the total bound C^{14}. The amino acids found in the hot trichloroacetic-acid-resistant residue (protein) and their respective activities are shown in Table I, while Fig. 2 indicates the percentages of C^{14}

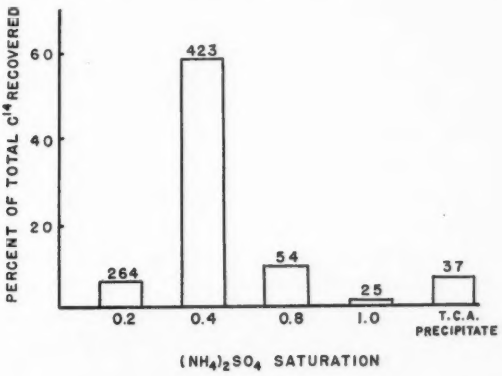

FIG. 2. C^{14} recovered in various protein fractions obtained from a sonic extract of C^{14}-labelled *R. meliloti*. The number on top of each vertical bar represents the specific activity of the fraction in counts per minute (above background) per mg dry weight. The T.C.A. (trichloroacetic acid) precipitate was obtained from the supernatant after removal of the last $(NH_4)_2SO_4$ fraction. The radioactivity present in the cellular debris is not included.

recovered in the protein fractions obtained from sonic extracts of labelled cells. A considerable amount of C^{14} was recovered in the heterogeneous residue obtained after initial centrifugation of the sonate, but this recovery is not included in the diagram.

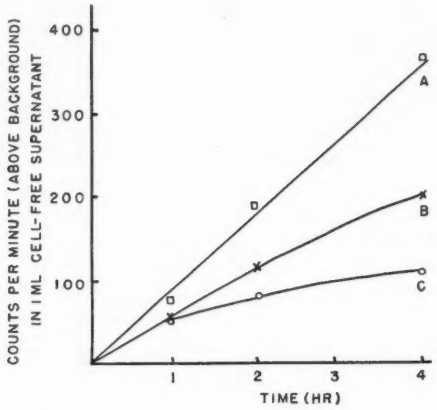

FIG. 3. Leakage of C^{14} compounds from equal amounts of non-growing cells of *R. meliloti* in glucose basal medium after prior incubation in medium containing DL-histidine-α-C^{14}. Curve A, X-irradiated cells, 30° C; curve B, non-irradiated cells, 30° C; curve C, non-irradiated cells, 5° C.

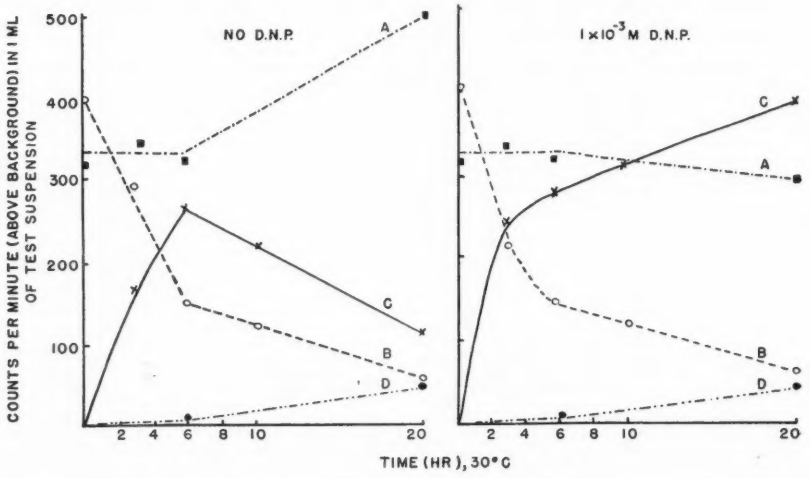

FIG. 4. Relationships of leakage C^{14}, pool C^{14} loss, bound C^{14} loss, and $C^{14}O_2$ production in labelled cells of *R. meliloti* incubated in the presence and absence of 2,4-dinitrophenol. Curve A, bound C^{14}; curve B, pool C^{14}; curve C, leakage C^{14}; curve D, respiratory $C^{14}O_2$ (minimum values, since some would be retained by the medium and contribute to leakage C^{14} values).

Washing labelled cells resulted in a loss of internal C^{14} amounting to as much as 30% or more of the original. Nevertheless, when washed cells were placed in a histidine-free basal medium containing glucose there was still a consistent initial loss of cellular C^{14} into the outside environment. This loss, which was associated with a decrease in pool C^{14}, occurred at both 30° C and 5° C and was accelerated by previous X-ray irradiation (Fig. 3). The leakage occurred both in the presence and absence of dinitrophenol for a period of at least 6 hours (Fig. 4); after this time growth took place in the absence of dinitrophenol, and a considerable amount of leaked material was recovered by the cells and incorporated, via the pool, into the bound form. From the 6th to the 20th hour the gain in bound C^{14} during growth was entirely balanced by the re-entry of leakage C^{14}. The over-all decrease in the pool level of the isotope during the same time interval was an apparent result of the increased rate of $C^{14}O_2$ production. When growth was prevented by the inhibitor the leakage continued after the 6th hour, but at a greatly reduced rate. During this inhibition the bound C^{14} was relatively stable for the initial part of the experiment, but a variable loss, amounting to about 8%, was apparent in some cases at the end of 20 hours, possibly as a result of cellular lysis.

After 3 hours at 30° C the identified leaked amino acids consisted of glutamate, serine, glycine, alanine, lysine, valine and/or methionine, and leucine and/or isoleucine. No histidine was located, but several ninhydrin-negative fluorescent compounds were found.

Discussion and Conclusions

Rhizobium meliloti contains an internal amino acid pool, and the accumulation of histidine and glutamate in this pool resembles the now classic examples described by Gale (9) for *Staphylococcus aureus*. The accumulation apparently occurs against a concentration gradient and is energy dependent, being inhibited by such inhibitors of phosphorylation coupling as azide and dinitrophenol. There is no suggestion in the present work, however, that the actual passage of the amino acid across the cell membrane is itself endergonic, since the amino acid may well be loosely bound by an endergonic binding process after a passive diffusion into the cell. If the latter case is correct, then the bonds formed must be extremely labile, being split by the hot water treatment used in the pool extraction.

The incorporation of C^{14} from the pool into various bound forms in *R. meliloti* appears to be, in part, an example of protein synthesis. It is inhibited by chloramphenicol (10, 11), and the nucleic-acid-free material can be hydrolyzed to constituent amino acids, many of which are radioactive. There is also little doubt that different proteins accept different amounts of labelled amino acids from the pool. The delay in the incorporation of pool C^{14} into protein until the pool C^{14} has reached a certain level perhaps indicates that all the required amino acids must be simultaneously present in sufficient amounts before incorporation can proceed (11, 16).

Although the major radioactive pool constituent in histidine-fed *R. meliloti* is histidine in the early stages of influx, the protein histidine is non-radioactive. The added histidine, therefore, is not incorporated directly into protein but is degraded and must be resynthesized. Unpublished experiments carried out over short time intervals indicate that the pathway of breakdown of histidine-α-C^{14} in this organism rapidly leads to the formation of C^{14}-glutamate, possibly via anaerobic deamination to urocanate. The constant metabolism of pool amino acids and their metabolic products explains the release of $C^{14}O_2$ in the present work, the endogenous respiratory quotient of 0.78 (15), and the endogenous formation of ammonia in resting cells of this organism (15).

There have been a number of recent reports concerning the leakage of nucleic acid (4, 17) and other intracellular components (5, 6) from microorganisms. Gale (8) found a small leakage of glutamate when cells of *Staphylococcus aureus* containing large amounts of several amino acids were incubated in an amino-acid-free medium, and he reported that this efflux was suppressed by the addition of glucose. Conversely, there was no outward migration of glutamate from *Streptococcus faecalis* unless glucose was present (7). It would appear that in the former instance leakage occurred because of an energy lack, whereas in the latter instance leakage was dependent upon a concurrent energy supply. With *R. meliloti* an efflux of nearly all the pool materials occurred, and the loss was completely energy independent, since it occurred both under a condition of glucose oxidation and when the supply of energy was presumably disrupted by the presence of dinitrophenol. The loss under these conditions, coupled with its appreciable occurrence at 5° C, indicates that the leakage in this case is probably a free diffusion effect. Therefore, the passage of certain amino acids across the cell barrier of this organism occurs by two methods. If the intracellular amino acids are considered as being free molecules, then an endergonic transportation is required for the influx and accumulation against a concentration gradient, while the efflux in the direction of the gradient occurs by what appears to be simple diffusion. In these respects the behavior of this organism somewhat resembles that of *E. coli* (3) and ascites carcinoma cells (13). The rapid loss of internal amino acids from this and other Gram-negative bacteria might explain the results of Taylor (18), who found free amino acids in Gram-positive bacteria only.

The promotion of leakage by X irradiation is interesting, but whether or not this phenomenon is primarily due to a direct interference with energy processes (1), to a destruction of the permeability barrier, or to some other process attendant upon cellular death is difficult to state.

It is worthy of note that when *Rhizobium* growth begins in a relatively restricted system, as is usual in in vitro experimentation, much of the leakage material initially lost is brought back into the cell and used for synthetic purposes. Under such conditions leakage does not prevent growth. Nevertheless, leakage may assume considerable importance in certain instances,

and consequently one must never assume that the biochemical potentialities of washed cells are identical in every respect with those of the same cells in an unwashed state.

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THE RELATION OF SELF-INHIBITION OF GERMINATION TO THE OXIDATIVE METABOLISM OF STEM RUST UREDOSPORES[1]

G. L. FARKAS[2] AND G. A. LEDINGHAM

Abstract

Studies were made on the oxidative metabolism of stem rust uredospores under self-inhibited conditions and when self-inhibition was removed. The oxidative metabolism of self-inhibited spores is characterized by the following features: the oxygen uptake declines rapidly; the respiration is mainly based on a fatty acid oxidation apparently bypassing the Krebs cycle (malonate insensitivity); and the enzymes of carbohydrate breakdown are more or less inactive. Compounds that are able to overcome self-inhibition stimulated oxygen consumption. Simultaneously an intensified utilization of endogenous fatty acids was initiated and also an increased malonate sensitivity. Longer incubation periods with stimulants resulted in germination and in a concomitant activation of carbohydrate metabolism. The catalytic effect of pelargonaldehyde or other stimulants in overcoming self-inhibition was reproduced by the administration in substrate concentrations of suitable respiratory material such as butyrate and propionate. The stimulated respiration induced by these substances appears to be a prerequisite for germination.

The possible significance of the above findings from the point of view of host–parasite relations is discussed.

Introduction

Germination of uredospores is governed by a balance between *stimulatory* and *inhibitory* substances contained by the spores or produced during germination (1, 13, 36). Substances of a similar nature might be present in the host tissues and play a definite role in host–parasite relations by affecting the first steps of rust development on or in the tissues. Therefore, a great deal of attention has been paid recently to the isolation and characterization of substances affecting spore germination.

Uredospores of wheat stem rust, when in contact with water, swell, respire, and produce one or more substances known as self-inhibitors that will completely suppress germination if present in sufficient amounts (1, 13). Compounds such as 2,4-dinitrophenol, menadione, coumarin, and certain of its derivatives, phenolic acids, β-indoleacetic acid, pelargonaldehyde (PA), and an unidentified factor extracted from cotton have been found to remove the inhibition when added to the medium (1, 2, 3, 15, 16, 32). Of these, coumarins, phenolic acids, and pelargonaldehyde have been found in uredospores (15, 32). Growth-promoting substances, possibly related to auxins, have also been extracted (26). All of these compounds affect oxidative metabolism or indirectly affect the energy-yielding processes of intermediary metabolism

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(4, 18, 20, 21, 35). Studies on the oxidative metabolism of uredospores and the effect of compounds that overcome self-inhibition thus appeared warranted. The present paper describes the alterations in metabolism induced by the application of substances that abolish self-inhibition.

Materials and Methods

Unless mentioned otherwise, freshly collected uredospores of stem rust (race 15B) were used throughout the experiments. They were produced in a temperature-controlled greenhouse on the wheat varieties Thatcher and Stewart. The spores were never stored in the refrigerator longer than 24 hours before being used for the assays.

Studies on the germination of spores were made as described previously (32). In the present paper the terms "self-inhibition", "self-inhibitor", "germination en masse", "spore traces", etc., are used according to the terminology introduced by Allen (1).

Standard manometric techniques were used in respiratory studies using a Warburg constant volume respirometer. Except as mentioned otherwise in the text, 5 mg spores per vessel was used in all experiments. Carbon-14 was determined by the method of Buchanan and Nakao (5).

All the experiments were repeated at least 4 times; in case of marked variation often up to 10 times. Averages or representative examples are given in the tables and figures.

Experimental

Studies on the Respiration of Spores

Previous studies in this laboratory demonstrated a surprisingly low respiratory rate for both resting and germinated uredospores (28). These results were confirmed under identical experimental conditions. However, the low rates observed are not an inherent characteristic of the spores but are due to several factors. First, the respiratory rate of older spores is significantly less than those freshly harvested. The consumption of oxygen by spores stored in a refrigerator for 7 days is less than that of fresh spores. After 1 month the rate was only 60% of that found for fresh spores and after 1 year only 29%.

In addition, the respiratory rate is not constant even for a short period (in contrast to the steady oxygen uptake of most other living materials rich in respiratory substrates), but decreases rapidly during the first 2 to 3 hours and reaches extremely low values in 12 to 16 hours (Fig. 1). It is unlikely that this decline is due to exhaustion of substrates since the spores are rich in a wide variety of respirable compounds including carbohydrates, fats, proteins (24, 30), and organic acids (33). Moreover, attempts to maintain oxidation at a steady rate by administering various carbohydrates and intermediates of the Krebs cycle failed.

It has been demonstrated that the amount of spores present in a given volume (water + air) greatly influences their ability to germinate (1). When

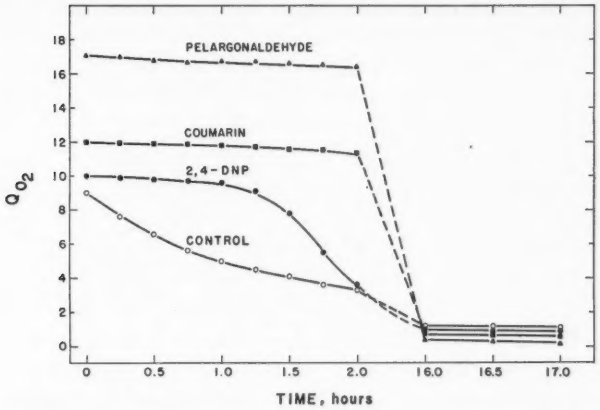

FIG. 1. Effect of compounds counteracting self-inhibition on the oxygen uptake of stem rust uredospores. Concentrations: 2,4-DNP, 10^{-5} *M*; coumarin, 5×10^{-4} *M*; pelargonaldehyde, 10^{-4} *M*.

the number of spores relative to volume is high, germination is low, caused by the accumulation of inhibitory substances. A similar relationship was obtained when the effect of increasing spore amount per volume on the O_2 uptake of spores was investigated. This is illustrated in Table I where higher respiratory rates are obtained when the ratio of spores to volume is low. Absorption of CO_2 by the 20% potassium hydroxide in the center well was not a limiting factor. The spores are apparently producing inhibitory substances. It is also interesting to note that the inhibition obtained was 50% of the initial respiration, regardless of the spore load.

If the reduction in respiratory rate is due to self-inhibition, compounds capable of overcoming self-inhibition should induce a rise in oxygen consumption. Several compounds known to counteract self-inhibition were tested and found to cause a consistent increase in oxygen consumption. Representative examples are shown in Fig. 1. Whereas the respiratory rate of the control spores declined rapidly, the rate of those treated with the compounds counteracting self-inhibition except 2,4-DNP was maintained on an increased level

TABLE I

Dependence of the absolute respiratory rate (Q_{O_2}) of stem rust uredospores on the relative amount of spores per vessel

	Amount of spores per vessel					
	2.5 mg		5 mg		10 mg	
	A	B	A	B	A	B
Q_{O_2}	10.6	5.3	9.6	4.8	7.5	3.9

NOTE: A = Q_{O_2} values at the beginning of the experiment.
B = Q_{O_2} values after 2 to 3 hours' incubation.

for several hours. Pelargonaldehyde was the most effective. After 3 hours the rates of the treated spores also declined and by 12 to 16 hours had reached the level of the untreated spores. The response to these different stimulators is dependent on the age of the spores, as the response with spores stored in a refrigerator for 2 to 3 months was usually less.

During these studies we observed that Seitz filters contain a substance that strongly stimulated respiration. The substance could be easily washed out of the filters and tested. The effect was similar to that of compounds counteracting self-inhibition. As the active substance could be extracted from practically any cellulose-containing material (filter paper sheets, powdered cellulose, cotton), it is probably identical with the "cotton factor" now being investigated by Wisconsin workers (3).

The Nature of Respiration in Resting and Germinating Spores

Previous studies in this laboratory have shown the presence of carbohydrate-metabolizing enzymes in stem rust uredospores (28). It was, therefore, rather surprising to find that a wide variety of carbohydrates and metabolic intermediates did not affect the endogenous respiratory rate (28) and were utilized to a very small extent when fed to the spores even though the necessary enzymes were present (28, 29). The spores preferentially utilized some endogenous substrate. These results were confirmed during the recent studies

TABLE II

Utilization of labelled substrates by stem rust uredospores with and without added pelargonaldehyde

	Control		Treated with 10^{-4} *M* pelargonaldehyde	
Added substrate	μM CO_2/5 mg spores/hour	% of $C^{14}O_2$ in respired CO_2	μM CO_2/5 mg spores/hour	% of $C^{14}O_2$ in respired CO_2
Sucrose-C^{14}	1.6	5.8	3.3	2.7
Acetate-1-C^{14}	1.5	3.3	2.8	1.8
Butyrate-1-C^{14}	2.0	31.9	3.2	17.9
Sucrose-C^{14}*	–	–	0.3	16.7

*Added after a preincubation with pelargonaldehyde for 16 hours.

TABLE III

The effect of fatty acids on the oxygen uptake and self-inhibition of spores

Substrate, 10^{-2} *M*	Increase in basal respiration upon addition of substrate, %	Germination 12 hours after treatment (germination en masse), %
Control	–	2
Acetate (C_2)	30	20
Propionate (C_3)	45	98
Butyrate (C_4)	60	99
Valerate (C_5)	49	3
Caproate (C_6)	65	4
Caprylate (C_8)	79	15
Pelargonate (C_9)	102	2
Palmitate (C_{16})	50	15

(Table II), except that acetate slightly stimulated the endogenous oxygen uptake (Table III) and a similar effect of succinate was experienced when higher concentrations were used (8×10^{-2} *M*). In some runs a slight stimulation with oxalacetate was also observed.

Since all these investigations were conducted under conditions of self-inhibition, the studies were repeated in the presence of PA (10^{-4} *M* in phosphate buffer, pH 6.1). Spores were floated in the main compartment of Warburg vessels on 10^{-4} *M* PA plus the labelled substrate (10^{-2}, 5×10^{-3} *M*) to be tested. The concentration of various labelled substrates in comparative experiments was chosen so that, as far as possible, the same amount of labelled carbon (micromoles) would always be present in the Warburg vessels. The radioactivity of the carbonate from the center well was measured. Results are expressed as per cent of respiratory carbon derived from labelled substrate.

As seen in Table II, when uniformly labelled sucrose was fed to untreated spores and spores treated with PA there was a strong respiratory stimulation induced by PA. The stimulation was not, however, associated with a concomitant release of higher amounts of $C^{14}O_2$ and even a smaller percentage of the sucrose was consumed by the treated spores than by the controls. This suggests that the stimulation induced by PA is not associated with increased sugar utilization. Similar data were obtained with acetate (Table II).

This conclusion was further substantiated by measurement of the respiratory quotient in self-inhibited and PA-treated spores. Although we experienced an unusually high variability between different runs under "identical" experimental conditions, it was evident that the R.Q. was not significantly altered during the first few hours in the presence of PA. The respiratory quotient in control and PA-treated spores was 0.65 and 0.67 respectively, indicating the utilization of fat as previously shown by Shu *et al.* (30). Fat utilization was also confirmed by chemical methods (7).

Labelled fatty acids were therefore tested as respiratory substrates. Experiments with butyrate-1-C^{14} indicated that resting spores are able to utilize fatty acids to a much higher degree than any other substrates tested. The average values are 10 to 20 times higher than those previously obtained with carbohydrates, volatile fatty acids, and amino acids in the presence or absence of PA (29).

Interestingly enough, although the respiration of spores treated with PA is apparently a fat respiration (R.Q. = 0.67), the $C^{14}O_2$ derived from the extraneously supplied butyrate is not increased, but (expressed in %) rather depressed. Therefore most of the additional CO_2, in the presence of PA, was evidently derived from some other endogenous source (Table II).

Other fatty acids, when tested as respiratory substrates on untreated spores, induced a 50 to 100% increase in oxygen uptake (Table III). Thus it appears that the normal metabolism of the spores, despite the presence of enzymes involved in carbohydrate metabolism, is a fat metabolism both in the inhibited state and during the first stages of germination. When the spores were allowed to respire in the presence of PA for 16 hours, the addition of

sucrose then resulted in a stimulation of oxygen consumption. Five times as much sucrose was oxidized to carbon dioxide during this period than when added before preincubation with PA (Table II). Thus the initial period of fat metabolism appears to change to one of carbohydrate metabolism. This is also indicated by a rise of R.Q. to 0.90.

The Significance of the Altered Metabolic Pattern for the Germination of Spores

Since we observed a qualitative difference in the malonate sensitivity of oxygen uptake in self-inhibited and PA-treated spores (Table IV), it was thought that the malonate-sensitive part of the respiratory pathway in PA-treated spores might be responsible for making germination possible. Indeed, malonate not only reduced the PA-induced respiratory increase but also inhibited the effect of PA as far as counteraction of self-inhibition is concerned. Therefore, the existence of a direct relationship between the malonate-sensitive part of PA-induced respiration and the germination process has been established (Table IV).

Interestingly enough, the malonate sensitivity of the oxygen uptake of PA-treated spores is only temporary (Fig. 2). Similarly, the inhibition of germination in the PA-treated spore samples by malonate is temporary. Actually, after an incubation period with PA + malonate for 12 to 14 hours, malonate stimulated germ tube growth compared with controls treated only with PA. It had no effect on self-inhibited samples (without PA treatment). In all experiments with malonate the system was buffered at pH 4.8 (also the controls).

TABLE IV

The effect of respiratory inhibitors on the oxygen uptake and germination of self-inhibited and pelargonaldehyde- and butyrate-treated spores

	Inhibition (−) or stimulation (+) of oxygen uptake, %			% germination after:					
				Treatment for 2 hours with:			Treatment for 16 hours with:		
Inhibitor	Control	Treated with 10^{-4} *M* pelargonaldehyde	Treated with 10^{-2} *M* butyrate	—	Pelargonaldehyde, 10^{-2} *M*	Butyrate, 10^{-2} *M*	—	Pelargonaldehyde, 10^{-4} *M*	Butyrate, 10^{-2} *M*
—	—	—	—	4	98	23	6	99	98
Malonate, 2×10^{-2} *M*	0	−36	−38	1	35	18	5	97	70
Hydroxylamine, 10^{-2} *M*	−25	−75	−67	0	1	0	1	2	2
Cyanide, 10^{-4} *M*	−98	−97	−95	1	2	2	3	1	3
Fluoroacetate, 5×10^{-2} *M*	0	0	0	3	90	18	3	95	92
2,4-Dinitrophenol, 5×10^{-4} *M*	+48	+2	+4	85	95	80	97	93	90

The above experiments with malonate are in agreement with the recent data of Staples (33) indicating a strong decrease of the endogenous malonate content of rust spores after germination has taken place. In all probability, malonate is a respiratory material for the spores in an advanced stage of germination. It is likely that the oxygen uptake of resting (self-inhibited) spores is malonate insensitive because of the presence of an established

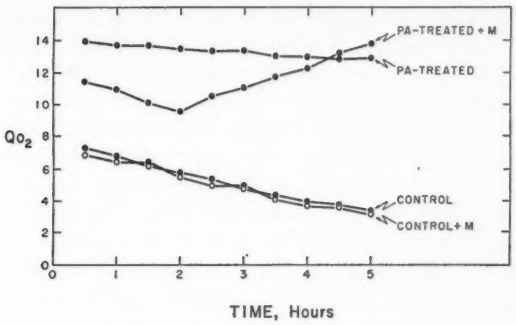

FIG. 2. Effect of 2×10^{-2} *M* malonate (M) on the oxygen consumption of control and 10^{-4} *M* pelargonaldehyde-treated (PA) stem rust uredospores.

endogenous malonate block. Succinate also (in the experiments described earlier) increased the oxygen uptake of spores by competitively removing the inhibitor. This view is supported by the observation that succinate increases the oxygen uptake only when applied in fairly high concentrations (up to 8×10^{-2} *M*).

In view of the above findings it seemed reasonable to conclude that a specific type of respiration is necessary for spore germination. It was likely that not only PA or other *stimulants* can shift metabolism in the required direction but the addition of a suitable *respiratory substrate* will also result in the overcoming of self-inhibition. To test a hypothesis that fatty acids, which stimulated oxygen consumption, would also overcome self-inhibition, the fatty acids were supplied in substrate concentrations (10^{-2} *M*). Some of these, in fact, induced almost 100% germination despite a spore load heavy enough to completely inhibit germination in controls (Table III). Butyrate and propionate were the most active compounds. In contrast to PA the fatty acids proved ineffective in catalytic concentrations (10^{-4} *M*). Thus the catalytic effect of PA and other stimulants could readily be reproduced by the application in higher concentrations of suitable respiratory substrates.

Certain fatty acids did not overcome inhibition despite their stimulatory effect on oxygen uptake. This seems to indicate that some specific deficiency exists in the process of catabolism of these fatty acids and in consequence of this no energy is provided in an adequate form for germ tube growth.

Experiments were conducted to show that the active compounds, for example butyrate, will exert a positive effect against self-inhibition only if oxidized through the cells. This was shown by the selective inhibition of butyrate oxidation. For example, whereas the basic respiration of the spores is not affected by malonate, the effect of butyrate on respiration is affected (Table IV). A similar pattern was observed with hydroxylamine, the control spores being less sensitive to it than the PA-treated ones. Both the basic respiration and induced respiration are totally sensitive to cyanide. The

inhibition of oxygen uptake with these inhibitors eliminated or at least hindered the effectiveness of butyrate in overcoming self-inhibition (Table IV). The effect of hydroxylamine was permanent. The spores later recovered from the malonate inhibition, i.e. the malonate effect was only temporary.

Experiments on the effect of pH on the butyrate-induced counteraction of self-inhibition also indicated that there is a direct correlation between the oxidation of butyrate and its ability to counteract the inhibitor. Spores did not oxidize butyrate at pH 4.5 to 5.0. When the oxidation of butyrate was inhibited by the low (but otherwise still physiological) pH, no germination took place. This may be related to the ionization constant of the fatty acids. Because of the pH sensitivity of butyrate oxidation, the experiments with butyrate plus malonate described above were carried out at pH 6.1.

Discussion

The peculiar properties of uredospore germination have attracted the attention of scientists for a long time. It became evident that the erratic germination, often leading to non-reproducible experimental results, is due to a highly sensitive interplay between stimulatory and inhibitory substances governing germination. The discovery of substances able to overcome the effect of endogenous germination inhibitors (2, 3, 15, 32) has an important implication in the better understanding of obligate parasitism. It was shown that the early developmental stages of the rust fungi are blocked by self-inhibitory substances and that the block can be eliminated by well-defined chemical entities. It is logical to hypothesize that the further developmental stages of the obligate parasitic fungi might be blocked in a similar way and are responsible for the failure to cultivate these microorganisms in vitro.

In the present study the counteraction of self-inhibition by any substance was invariably associated with a marked but temporary respiratory increase preceding germination. This suggests that the increased metabolic activity induced by the "counteracting" compounds is a prerequisite of germination, particularly as butyrate and propionate first stimulate respiration, then germination.

It seems likely that the enzyme system activated by PA in PA-treated spores is identical with the system utilizing added butyrate in the spores since: (*a*) the endogenous oxygen uptake of PA-treated spores (unlike that of the inhibited ones) is not further increased by added butyrate; (*b*) both the butyrate- and PA-stimulated respiration of spores is sensitive to malonate, in contrast to the malonate-insensitive endogenous respiration of untreated spores; and (*c*) both the PA- and butyrate-stimulated respiration is equally highly sensitive to hydroxylamine, whereas the oxygen uptake of untreated spores is strongly hydroxylamine resistant (Table IV).

Some information has been obtained as to the nature of the metabolic block. During swelling and the first hours of germination, the spores preferentially utilize fatty acids as respiratory substrates. Since butyrate and propionate stimulate respiration and germination, the utilization of endogenous

fatty acids is apparently blocked in the self-inhibited spores. The substances capable of breaking this block belong to the group of uncouplers (2,4-DNP, menadione, phenol derivatives). The other stimulators might be uncouplers as well but have not been investigated from this point of view. The inhibitor may be formed by some process involving phosphorylation which is more sensitive to uncouplers than other phosphorylations of vital importance to germ tube growth. The differential sensitivity of various phosphorylation steps towards uncouplers is a well-established fact (4, 9, 20, 31).

As indicated, the inhibitor behaves in many respects like a respiratory poison, the effectiveness of which is nullified by uncoupling. It is known that in some cases respiratory poisons must be "activated" before they become effective and this activation is an energy-requiring process. The best-known example is the activation of fluoroacetate which takes place via fluoroacetyl-CoA, followed by the formation of fluorocitrate that inhibits the aconitase system (6, 23). The toxicity of fluoroacetate can be overcome by 2,4-DNP (17) which prevents the activation of fluoroacetate by CoA and ATP, or by addition of a surplus of C_2 units (23) (competitive antagonism). By analogy the same appears true of the self-inhibition of rust uredospores (30). The inhibition can be overcome by uncouplers and also by the administration of various fatty acids yielding C_2 units. The insensitivity of oxygen consumption by uredospores to fluoroacetate (which is a phenomenon highly uncommon in living systems) can be interpreted as indicating that a substance having similar properties is already present.

The data are therefore consistent with the working hypothesis that the point of attack on the inhibition may be the CoA – fatty acid complex. Removal of this inhibition leads to an enhanced operation of the Krebs cycle. Some earlier observations by Allen (1) that it is easier to overcome self-inhibition when the carbon dioxide tension is high can be interpreted in this manner. We confirmed his results and also observed that the beneficial effect of CO_2 can partially be replaced by oxalacetate in a CO_2-free atmosphere (12). That carbon dioxide is incorporated into the Krebs cycle has been demonstrated by studies on bean rust with isotopic carbon (34).

There are, of course, several questions that need further explanation. One of these is the peculiar fact that acetate is only slightly effective in stimulating respiration and overcoming self-inhibition. However, the enzymes of fatty acid activation are highly specific (14, 19). In rust spores we apparently are concerned with an enzyme that activates propionate and butyrate rather than acetate (22).

Another problem is why some fatty acids stimulate the respiration of spores but do not affect self-inhibition. The most plausible explanation we can offer is that the breakdown of these fatty acids does not take place via the CoA $\longrightarrow$ Krebs cycle (β-oxidation) pathway (which seems to be decisive for germination) but possibly via the α-oxidation pathway bypassing the Krebs cycle (14). Unfortunately the malonate sensitivity of the extra

respiration induced by these acids could not be properly tested because the higher members of the series are insoluble at lower pH (required by the malonate assay) and the lower members are not oxidized when the pH is around 4.5–5.0.

Last but not least we have to emphasize the finding that, after germination has taken place, the spores exhibit a trend toward a carbohydrate type of respiration. At this developmental stage both fatty acids and sugars are utilized if supplied to the spores, in contrast to the preferentially lipid type of metabolism at the beginning of swelling and germ tube growth. This makes it likely that in the host tissues the rust metabolism is basically a carbohydrate metabolism. This switch in the type of metabolism seems to be of paramount importance from the point of view of host–parasite relations. Parasitism, especially in the obligate form, is certainly based (at least in the initial stages of disease development) on the harmonic co-operation of the metabolic pathways of the two organisms. This evidently cannot take place without some rearrangement in the metabolism of the host–parasite complex and such has been shown to occur in the host (8, 10, 11, 25, 27). The present paper shows that the initial lipid type of metabolism in the rust uredospore changes to a carbohydrate type of metabolism during germination and this apparently makes it possible for rust to take advantage of the stimulated carbohydrate accumulation and breakdown in the host.

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THE METABOLISM OF YEAST SPORULATION

III. RESPIRATION OF SPORULATING AND GROWING CELLS[1]

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Abstract

Cells from growth medium had a strong endogenous respiration under aerobic conditions with a respiratory quotient of approximately unity. In *M*/300 glucose, their oxygen uptake was somewhat greater than the endogenous, but the aerobic carbon dioxide output was approximately twice the oxygen uptake. When such cells were incubated in buffer no change in their respiration in glucose was noted in 2 days, but when incubated in 0.3% acetate the respiratory quotient declined to 1.3–1.5. This decline was evident within six hours of the time the cells were placed in acetate. Glucose (0.1%) also depressed the respiratory quotient. With two other sporulation substrates, sodium pyruvate (0.13%) and lactic acid (0.1%), the effect was not so pronounced, and a fifth, dihydroxyacetone (0.1%), seemed to have little or no effect. Spores developed more rapidly and became more abundant in the acetate than in any of the other compounds. The changes in the respiratory quotient did not show a clear correlation with either the amount or the rapidity of sporulation in the five sporulation substrates. When cells were incubated in acetate or in glucose for a day under anaerobic conditions their respiratory quotient did not decline. Some success was obtained in separating sporulated from non-sporulated cells by centrifuging. No difference was noted in the respiration of sporulated and non-sporulated cells. The respiratory quotient of cells from sporulated cultures returned to values characteristic of growing cells after 2 to 4 hours in growth medium.

Introduction

Although the carbohydrate metabolism of *Saccharomyces cerevisiae* has been intensively investigated, very little attention has been given to the respiratory changes that occur in cells transferred from a growth medium to an environment in which they will form ascospores. The published observations are, moreover, of an indirect nature. Thus Bautz (1) in a study of the cytological effects of the Nadi reagent found evidence of a strong oxidizing activity in sporulating cells. Miller and Halpern (4) observed strong inhibition of sporulation by cyanide and fluoroacetate. There is, moreover, general agreement (5) that aerobic conditions are essential for sporulation. The preceding papers of this series summarize earlier work by various investigators in which it has been established that the only exogenous nutritional requirement for sporulation of "well-nourished" cells of certain yeast strains is a carbon source (sugars or acetate) in appropriate concentration. Miller and Halpern (4) found that three other intermediate products of carbohydrate metabolism, pyruvate, acetaldehyde, and ethanol, supported sporulation. The purpose of the present study was to compare the respiratory activity of sporulating cells with that of growing cells. It seemed reasonable to anticipate that some evidence would thereby be found of biochemical differences between sexually and asexually reproducing yeast cells.

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Materials and Methods

Ideally the respiration of cells in sporulation medium should be compared with that of cells actively growing in a nutrient medium. However, differences in oxygen uptake or carbon dioxide output under these circumstances could conceivably be the result of differences in the chemical environments presented by the two media. For this reason it was deemed advisable to remove the cells from these media and observe the effect of the pretreatment on their respiration in Warburg vessels containing a solution of glucose in buffer.

The yeast was grown in yeast nitrogen base (Difco). This was dissolved in buffer with 1% glucose added for the carbon source and placed in 10 ml quantities in pharmaceutical bottles of approximately 100 ml volume. Following inoculation the bottles were incubated at 27° C on their flat sides to aid aeration.

Cells were separated from 2-day growth cultures by centrifugation, washed three times in buffer, and resuspended in sporulation medium at a cell population density of 4 million per ml. This was dispensed in 100 ml volumes in 1-liter culture flasks which were placed on their flat sides in the incubator at 27° C and gently shaken during the period of incubation to prevent the cells from settling out and to ensure thorough aeration. The sporulation medium consisted of buffer containing 0.3% sodium acetate ($CH_3COONa.3H_2O$), or 0.1% glucose, or 0.13% sodium pyruvate, or 0.1% lactic acid, or 0.1% dihydroxyacetone as the sporulation substrate.

Manometric experiments were done at 27° C under aerobic and anaerobic conditions following the standard techniques (8). The total volume of liquid in the Warburg vessels was 3 ml (1.9 ml buffer + 1 ml yeast suspension containing 12 million cells, in buffer, + 0.1 ml of $M/10$ glucose solution in the sidearm). The final concentration of glucose in the vessels was 0.06%. This rather dilute solution was considered appropriate because it approximates the optimum concentration of glucose for sporulation as determined in a preceding investigation (3). The yeast suspensions were prepared with cells that were separated from growth or sporulation media by centrifuging and washed three times in buffer. Respiration measurements were made for 2 hours after the contents of the sidearm were mixed with the other contents of the vessel. The buffer used throughout was $M/30$ phthalate of pH 5. Other techniques were as previously described (3, 4). The yeast culture was an isolate from Fleischmann baking yeast (*Saccharomyces cerevisiae*) designated as F49.

Results

Endogenous Respiration of Growing and Sporulating Cells

Table I summarizes the results of several experiments designed to assess the extent of the endogenous respiration in growing and sporulating cells. It is evident that the growing cells had a strong endogenous respiration under aerobic conditions, and that this showed some decline after 2 and 3 days

in acetate sporulation medium. Anaerobic respiration seemed slight and the respiratory quotient approximated unity, which are features usually observed in the endogenous respiration of yeast (2).

TABLE I

Endogenous respiration of growing and sporulating cells

Treatment of cells	Sporulation, %	Oxygen uptake, μl in 2 hr	Aerobic carbon dioxide production, μl in 2 hr	Anaerobic carbon dioxide production, μl in 2 hr	Respiratory quotient*
Growing	0, 0, 0	45, 49, 61	48, 49, 74	5, 0, 0	1.1, 1.0, 1.2
1 day in buffer	0	48	48	0	1.0
2 days in buffer	0	34	34	0	1.0
1 day in acetate solution	15.5, 8.5	41, 37	42, 37	10, 0	1.0, 1.0
2 days in acetate solution	43, 30	27, 21	29, 21	6, 0	1.1, 1.0
3 days in acetate solution	51, 47	23,15	29, 15	3, 0	1.3, 1.0

*R.Q. $= \frac{\text{aerobic carbon dioxide production}}{\text{oxygen uptake}}$.

Respiration of Growing Cells in Glucose

Cells transferred directly from growth cultures to the Warburg vessels (Table IIA) had an oxygen uptake somewhat greater than the endogenous respiration of similar cells shown in Table I. The carbon dioxide production under aerobic conditions was, however, much greater than the oxygen uptake, indicating a strong aerobic fermentation. A high respiratory quotient and active anaerobic respiration were characteristic of the growing cells.

Growing cells after up to two days in 1-liter culture flasks containing 100 ml of buffer, gently shaken as with sporulation cultures, showed little or no change in respiratory activity, and the respiratory quotient remained high.

Respiration of Sporulating Cells in Glucose

When cells were transferred from growth medium to acetate sporulation medium, a distinct decline in the respiratory quotient became apparent within four to six hours (Table IIB). The quotient also declined in glucose and in lactic acid sporulation media, but in pyruvate the decline was less rapid. Dihydroxyacetone had comparatively little effect on the respiratory quotient. The changes in the respiratory quotient did not seem to have any direct correlation with either the rapidity or the amount of sporulation observed in these five substrates.

In most sporulation media the ability to consume oxygen in glucose diminished, but a correspondingly greater diminution in the carbon dioxide output was responsible for the decline in the respiratory quotient.

The possibility that the respiratory quotient would rise again if the cells were transferred to buffer from glucose or acetate solutions was investigated in the following manner: cells were shaken for 1 day in acetate and in glucose solutions in the usual way. They were then recovered by centrifugation,

TABLE II

Respiration of growing and sporulating cells in glucose

Treatment of cells	Number of determinations	Sporulation, %	Oxygen uptake, μl in 2 hr	Aerobic carbon dioxide production, μl in 2 hr	Anaerobic carbon dioxide production, μl in 2 hr	Respiratory quotient*
A. Growing	12	0	63†	140†	97†	2.2
4 hours in buffer	6	0	45	104	95	2.3
1 day in buffer	3	0	61	130	101	2.1
2 days in buffer	2	0	57	120	83	2.1
B. ½ hr in acetate solution	1	0	48	124	122	2.6
1 hr in acetate solution	2	0	68	164	143	2.4
2 hr in acetate solution	3	0	79	145	107	1.8
4 hr in acetate solution	3	0	72	121	96	1.7
6 hr in acetate solution	1	0	69	104	55	1.5
1 day in acetate solution	7	6	46	60	34	1.3
2 days in acetate solution	5	36	36	52	25	1.4
3 days in acetate solution	5	51	28	40	16	1.4
1 day in glucose solution	2	0	34	38	17	1.1
2 days in glucose solution	4	8	32	40	19	1.25
3 days in glucose solution	2	21	34	41	8	1.2
1 day in pyruvate solution	2	1.5	66	134	99	2.0
2 days in pyruvate solution	2	9	56	95	83	1.7
3 days in pyruvate solution	2	24	55	76	60	1.4
1 day in lactic acid solution	2	0.5	60	91	77	1.5
2 days in lactic acid solution	2	29	43	59	49	1.4
3 days in lactic acid solution	2	44	31	48	36	1.55
1 day in dihydroxyacetone solution	2	0.5	68	142	104	2.1
2 days in dihydroxyacetone solution	2	8.8	49	88	59	1.8
3 days in dihydroxyacetone solution	2	14	41	61	37	1.5
C. 1 day in acetate solution; 1 day in buffer	1	23.5	33	35	21	1.1
1 day in glucose solution; 1 day in buffer	1	2	31	32	7	1.0
D. 1 day in acetate solution, anaerobic	1	0	30	67	57	2.2
1 day in glucose solution, anaerobic	1	0	23	48	25	2.1
E. 3 days in acetate solution; 1 hr in growth medium	2	69	38	55	54	1.45
3 days in acetate solution; 2 hr in growth medium	2		31	55	72	1.8
3 days in acetate solution; 4 hr in growth medium	2		53	145	141	2.7

*R.Q. $= \frac{\text{aerobic carbon dioxide production}}{\text{oxygen uptake}}$.

†The values for oxygen uptake and carbon dioxide production are not corrected for endogenous respiration.

suspended in buffer, and shaken in the incubator for a second day. The results of the manometric experiments, shown in Table IIC, indicate that the respiratory quotient after this treatment was still low.

Respiration of Cells from Sporulation Media Kept Anaerobic

It seemed of interest to determine whether the respiratory quotient would decline under anaerobic conditions. Cell suspensions in acetate and glucose sporulation media were shaken for 1 day in the usual manner, but the air in the 1-liter culture flasks was first replaced by oxygen-free nitrogen. In neither instance was a respiratory quotient of less than 2 observed (Table IID).

Respiration of Sporulated Cells after Transfer to Growth Medium

The possibility of raising the low respiratory quotients of cells from acetate sporulation medium by placing them in growth medium was investigated. Cells that had been shaken for 3 days in the sporulation medium were recovered by centrifugation, resuspended in growth medium, and shaken in 1-liter culture bottles. Samples were removed after 1, 2, and 4 hours for manometric study. The results of the two experiments (Table IIE) show that within four hours the respiratory quotient had risen to the high values characteristic of growing cultures. Observations on the germination of sporulated cells have shown that 3 hours after transfer to growth medium in shaken flasks a few spores are beginning to germinate, and after 4 hours approximately one fifth have done so. The non-sporulated cells in the suspension begin to grow sooner; that is, in about two hours.

Respiration of Spores

The spores of *Saccharomyces* cannot readily be separated from the surrounding ascus wall. This makes it difficult to measure their respiration since functional respiratory enzymes may be present in the epiplasm of the ascus. Moreover, not all the cells in a culture sporulate and the relative contributions by sporulated and non-sporulated cells to the net respiration cannot be determined.

Some success has been obtained in separating sporulated from non-sporulated cells by centrifugation. The former tend to be slightly heavier, and by five or six centrifugings of a sporulated suspension, each time skimming off the superficial cells of the centrifugate, it has at times proved possible to raise markedly the proportion of sporulated cells in a given suspension. Results with this method have, for some reason, not been consistent, and sometimes little or no change in the proportion of sporulated cells was observed after repeated centrifugings.

On one occasion a suspension of cells after 3 days in acetate sporulation medium contained 61% sporulated cells. After six centrifugings 93% of the cells that remained contained spores whereas only 27.5% were found among the cells that had been skimmed off. The proportion of asci containing three or four spores was lower in the latter than in the remainder of the centrifugate. When the respiration of the cell suspensions containing 93,

61, and 27.5% spores was compared, their rates of oxygen uptake were 27, 26, and 26 μl respectively. That is, the sporulated cells seem to have respired as actively as the non-sporulated. All consumed oxygen at about half the average rate observed above (Table IIA) with growing cells.

Discussion

As pointed out earlier in this series (4) the conversion of yeast cells from the growing to the sporulating condition must involve biochemical changes of great biological importance because it implies that nuclei cease to divide equationally and instead division becomes reductional. In so far as the authors are aware, no previous measurements have been made of the respiration of yeast cells during the transition from growth to sporulation.

It is of interest that Slonimski (6) noted that the respiratory quotient of yeast cells respiring in glucose solution in Warburg vessels fell from values of 1.5–2.0 to 1.0 within three hours. The most marked physiological change observed by the writers in cells sporulating in acetate or glucose was a decline in the value of the respiratory quotient. This was not found with cells suspended in buffer for an equivalent length of time, and since sporulation did not occur in buffer without an appropriate carbon source, one could speculate that the low respiratory quotient is in some way associated with sporulation. Since the decline precedes the first appearance of spores it might be a prerequisite for their formation, and this is also suggested by the failure to observe a change in the respiratory quotient of growing cells placed in sporulation medium under anaerobic conditions, which do not allow sporulation. However, when dihydroxyacetone served as the sporulation substrate, sporulation occurred without a definite decline in the respiratory quotient, so this change is evidently not essential for sporulation although conceivably it may be in acetate or glucose. Dihydroxyacetone has previously been found to have some unique features as a sporulation substrate (3).

A basic problem in yeast sporulation is the function of the sporulation substrate. Its most probable functions are (*a*) the supplying, through respiration, of energy-rich molecules that make possible synthetic reactions necessary for spore formation, or (*b*) serving as a precursor for the synthesis of compounds required in sporulation. With regard to the former, the cells of this strain of yeast showed an active endogenous respiration, and the oxygen uptake was only about one fifth greater when glucose was present in the Warburg vessels in a concentration (0.06%) approximating the optimum for sporulation. Thus, it appears quite possible that the energy requirements for sporulation could be met by the endogenous respiration of the cells. This is made more plausible by the results of several 2-hour experiments in which acetate, or pyruvate, or dihydroxyacetone served as the substrate in the Warburg vessels at concentrations which, though low, were sufficient to support sporulation. Evidence for oxygen uptake in excess of the usual endogenous value was not obtained. In this connection it is of interest to recall that Stantial (7) obtained good sporulation with cells exposed to acetate

and glucose for only 6 hours and then transferred to distilled water. A respiration of these substrates well in excess of the endogenous would seem necessary if such a short exposure is adequate. These experiments indicate that careful study should be given to the possibility that the sporulation substrates serve as precursors for compounds essential in sporulation.

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THE PRODUCTION BY BACILLUS THURINGIENSIS BERLINER OF A HEAT-STABLE SUBSTANCE TOXIC FOR INSECTS[1]

ELLICOTT MCCONNELL[2] AND A. GLENN RICHARDS

Abstract

Bacillus thuringiensis Berliner produces in vitro a heat-stable, dialyzable substance which is toxic for insects when injected. The same or a similar substance is produced in vivo. The toxic principle is of unknown composition. It is heat-stable, water-soluble, dialyzable, and resistant to low temperatures. It is probably neither a protein nor a lipid. Clearly it is distinct from the heat-labile inclusion bodies and from lecithinase. Growth-curve studies showed that the heat-stable toxin appeared in liver broth cultures during the active growth phase, prior to the formation of spores or inclusion bodies. An attempt to produce the toxic principle from culture media in the absence of bacteria was unsuccessful from sterile inocula both from in vivo and in vitro sources. The LD_{50} for larvae of *Galleria mellonella* injected with autoclaved supernatant from a 10-day-old liver broth culture of *B. thuringiensis* was determined to be 0.00036 ml per larva or 0.002 ml per gram of larvae. Approximately the same level of toxicity was found for another caterpillar, a fly larva, and cockroaches. After larvae of *Galleria* or *Pyrausta* have been dead for more than 2 days from infection with *B. thuringiensis* the bacillus could no longer be recovered. A sublethal amount of the heat-stable toxin injected into old larvae of *Galleria* delayed emergence of the adults by 30 to 40%. The non-pathogenic *Bacillus cereus* was found to produce a similar-acting, heat-stable toxin under the same conditions that one is produced by *B. thuringiensis*.

Introduction

The bacterium *Bacillus thuringiensis* and related forms have been considered among the most promising of the bacteria for the purpose of biological control of insect pests. Briefly, they are pathogenic for a number of insect species, are easily grown on artificial media without loss of virulence, and are capable, as sporeformers, of enduring for years in storage.

Although field experiments have not been neglected, there has been a recent tendency to emphasize research on specific aspects of the relationship of these bacilli to their environment. Among the leaders in these investigations are Steinhaus and his associates in California, and Angus, Hannay, and Heimpel in Canada. Their work indicates that the toxicity of *B. thuringiensis* for insects is a complex phenomenon. Two thermolabile toxic substances have already been demonstrated.

In the course of laboratory studies here, the production in vivo by *B. thuringiensis* of a heat-stable substance which killed insects upon injection was discovered. A study of this new toxin is the subject of the present paper.

The original thesis, on file in the University of Minnesota library, gives full tabular data on all the experiments, as well as a review of the literature.

[1]Manuscript received November 17, 1958.
Contribution from the Department of Entomology and Economic Zoology, University of Minnesota, St. Paul, Minn. Paper No. 4075, Scientific Journal Series, Minnesota Agricultural Experiment Station. Condensation of a thesis submitted by E. McConnell to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree Doctor of Philosophy.
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Materials and Methods

The culture of *B. thuringiensis* used was obtained from Dr. E. A. Steinhaus. Broth cultures were grown in Difco nutrient broth containing 10%, by volume, liver infusion. The infusion was prepared by autoclaving 1 lb of chopped beef liver in 1 liter of distilled water, followed by filtration through filter paper. The enriched medium, designated as liver broth, promotes more abundant growth than plain nutrient broth does. Subculturing of all species of bacteria used in this work was carried out on Difco nutrient agar.

As test insect, most of the work was done with mature larvae of the wax moth, *Galleria mellonella* (L.). These larvae are readily cultured throughout the year, are large enough to manipulate easily, have little tendency to lose fluid postinjection, and are not susceptible to natural infection. The larvae were reared at 35° C on either Haydak's mixture or on a 1:1 mixture of ground dog food and Gerber cereal food wet to the consistency of wet sand with a 3:1 mixture of honey and glycerine. Other species of insects, when used, were taken from stock laboratory cultures.

Injections were standardized at a volume of 0.01 ml, with the dosage being controlled by dilution of the inoculum being tested. Injections were made with a fine hypodermic needle in the posterior pleural region with the needle projecting anteriorly for at least $\frac{1}{4}$ in. past the insertion point. After injection, larvae were placed on filter paper in petri dishes for observation.

Results

Production of the Toxin

Demonstration of the presence of a heat-stable toxic factor is simple if one uses fairly concentrated filtrates and observes the animals for a week or more. For instance, in one test, 15 *Galleria* larvae were killed by injection with *B. thuringiensis* and 36 hours later homogenized in 10 ml distilled water in a Waring Blendor. The homogenate was filtered through an ultrafine fritted glass filter and then divided into two parts. One part was autoclaved at 15 lb pressure (= 121° C) for 15 minutes, the other was not heated. On injection into healthy larvae the two fractions gave comparable mortality (Table I).

TABLE I

Injection of *Galleria* larvae with heated and unheated filtrates of *Galleria* larvae dead from infection with *B. thuringiensis*

Inoculum	Milliliters injected	No. of larvae	No. of survivors
Autoclaved filtrate	0.02	5	0
	0.01	5	0
	0.005	5	0
	0.001	5	5
Unheated filtrate	0.02	5	1
	0.01	5	0
	0.005	5	1
	0.001	5	5
Filtrate from healthy larvae	0.02	5	5

A similar-acting factor—presumably the same factor—is produced in vitro. For instance, liver broth and nutrient broth cultures of *B. thuringiensis* incubated for 10 days at 30° C, autoclaved, filtered, and injected into *Galleria* larvae showed considerable toxicity. The filtrate from the liver broth culture was more potent, probably because of heavier bacterial growth in this medium. It was similarly shown that the bacterium growing on solid nutrient agar medium also produces the toxin. In other words, the toxin is a characteristic by-product of growth of this bacterium irrespective of how the bacteria were grown.

The relationship between the growth of *B. thuringiensis* and the production of the heat-stable toxin was studied in several ways. The most successful method involved 500 ml liver broth cultures incubated at 30° C on a mechanical shaker. Several such runs were made under similar conditions, and conventional methods were used to construct a growth curve. Simultaneously, aliquots were used to bio-assay for the concentration of heat-stable toxin. These data show that the heat-stable toxin parallels cell growth, and reaches a maximum within 24 hours (Fig. 1, Table II). Since the formation of spores and inclusion bodies occurred almost entirely in the interval 30–36 hours, this is clear evidence that the heat-stable toxin is not related to sporulation and not related to the visible inclusion bodies.

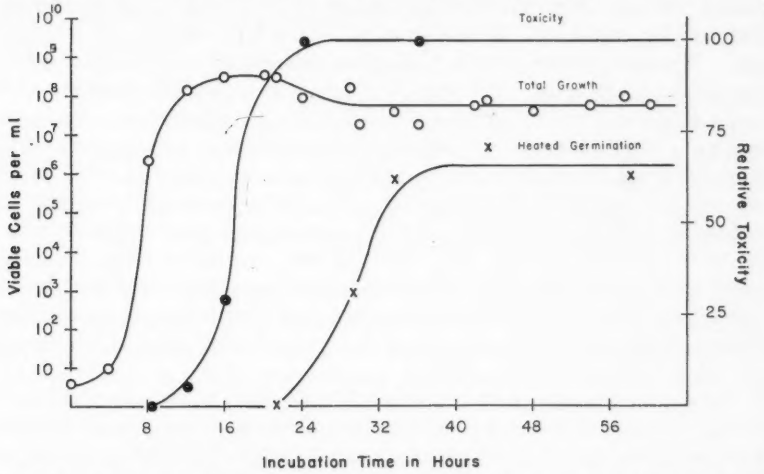

FIG. 1. The growth of *B. thuringiensis* cultured in liver broth related to the toxic effect of the autoclaved supernatant when injected into *Galleria* larvae.

Incidentally, in our culture of *B. thuringiensis* there is a striking difference depending on whether the broth culture is shaken or not. Beginning at about 30 hours after incubation, almost all cells formed spores and inclusion bodies in the shaken cultures. But in unshaken cultures hardly 1% of the cells formed spores; the remainder of the cells slowly formed prespores (reaching a maximum after 7 to 10 days) that did not develop further. But both

shaken and unshaken cultures produce the heat-stable toxic. This is additional evidence that production of the heat-stable toxin is not associated with spore formation. Seemingly, aeration is necessary for sporulation but not for production of this toxin.

TABLE II

Production of heat-stable toxic fraction, as a function of culture age, in a liver broth culture of *B. thuringiensis* incubated on a shaker at 30° C. Values given are number of survivors out of 10 larvae injected with each dilution

Milliliters injected	Age of culture in hours								
	0	4	8	12	16	24	36	72	96
0.02	10	10	8	6	0	0	0	0	–
0.01	10	10	10	9	2	0	0	0	–
0.001	–	–	10	10	10	0	0	0	1
0.0001	–	–	10	9	10	10	10	10	10
0.00001	–	–	–	10	–	10	10	10	10

The inability of unshaken cultures to sporulate may be related to a chance observation in vivo. Upon attempting serial passage of *B. thuringiensis* through *Galleria* larvae, we seldom recovered the organism after more than 2 or 3 days post-mortem. This was true for larvae of both *G. mellonella* and *Pyrausta nubilalis* infected either by feeding or by injection. Recovery was attempted by streaking the contents of infected larvae on nutrient agar plates. Typical profuse growth of *B. thuringiensis* occurred on plates from larvae dead for about 24 or 48 hours, but from larvae dead for longer periods only an occasional typical colony of the bacillus would appear. Numerous colonies of small rods or cocci, possibly enteric organisms, grew on some of the plates, but these are readily distinguished from *B. thuringiensis*. The failure to recover the bacillus was unexpected. Mattes (6) reported heavy sporulation following death of the host. In the present case there must have been little or no sporeformation, and death of the vegetative cells. It seems possible that a self-inhibitory substance might have been produced by the bacillus, but tests intended to demonstrate that the development of a self-inhibiting substance gave only negative results. Further study is needed to elucidate this point.

It can be readily shown that the heat-stable toxin is associated with the bacteria, and is not just some extracellular product of the culture medium. A liver broth culture of *B. thuringiensis* was incubated on a shaker at 30° C. Samples were taken at the end of 1 and 4 days. Part of each sample was filtered, and the filtrate was used to inoculate a sterile liver broth medium which was then incubated. Filtrates from the original culture and the subsequent incubation were then autoclaved and bio-assayed with *Galleria* larvae. When the toxicity of the original filtrates is known it is possible to calculate what toxicity is to be expected following the dilution involved in making the subsequent sterile inoculations. The bio-assay data are consistent with the view that the heat-stable toxin is produced only in the presence of living and growing bacteria.

Properties of the Heat-stable Toxin

The toxin was not identified chemically but some of its properties were determined. Dialysis of an autoclaved culture for 48 hours against distilled water showed the toxin to be dialyzable. Actually, bio-assay of the dialyzate shows that dialysis continues for days but the experiments performed do not show whether the slow removal is due to diffusion difficulties, elution, or something else, such as the separation of a prosthetic group. The demonstration of dialysis does, however, imply a relatively small molecule whatever the reason for the slowness of its removal.

The addition of an equal volume of 10% trichloroacetic acid added dropwise to a concentrated diffusate produced no visible effect. Simple distillation of diffusate at approximately 100° C simply concentrated the toxin which remained behind in the distillation flask. Shaking a concentrated diffusate from an autoclaved and dialyzed culture with petroleum ether in a separatory funnel revealed, after bio-assay, that the toxin remained in the aqueous phase. Finally, within experimental error, no decrease in toxicity occurred in an autoclaved filtrate following a 12-month storage at −20° C.

Summarizing: The heat-stable toxic substance produced by growing *B. thuringiensis* is a water-soluble, dialyzable, non-volatile compound which is also resistant to low temperatures. Attempts at precipitation and fractionation imply that it is neither a protein nor a lipid.

The Level of Toxicity of the Heat-stable Toxin

Since the toxin has not been isolated, values can be stated only in terms of some standard preparation. A 500-ml liver broth culture of *B. thuringiensis* was incubated at 30° C for 10 days. The culture was not shaken mechanically, but was occasionally swirled by hand to break up any pellicle which may have formed. At the end of the incubation period the culture was autoclaved at 15 lb pressure for 15 minutes and centrifuged to remove cell debris.

Preliminary injections with similar cultures had indicated that the LD_{50} for *Galleria* larvae would lie between 0.001 ml and 0.0001 ml of the supernatant. To obtain a more precise determination, 120 *Galleria* larvae of uniform large size were injected amounts representing 0.0001 to 0.0010 ml at 0.0001 ml unit intervals. The indicated LD_{50} is 0.00036 ml of supernatant per larva, or 0.002 ml per gram of larvae.

A few tests were made with relatively small numbers of other species of insects. The data suggest that the LD_{50} for injections with this inoculum do not differ greatly in different species of insects. Species tested in addition to *Galleria* were the European corn borer (*Pyrausta nubilalis*), a blowfly larva (*Sarcophaga bullata*), and two species of cockroaches (*Periplaneta americana* and *Blatta orientalis*).

Comparison with Bacillus cereus

The pathogenicity of *B. thuringiensis* for insects has been considered a diagnostic characteristic (Steinhaus (8)). It seemed desirable, therefore,

to determine if the ability to produce a heat-stable toxic substance is restricted to this organism. Accordingly, *B. thuringiensis* was compared with a strain of *B. cereus* previously found to be non-pathogenic for insects (McConnell and Cutkomp (7)).

The results presented in Table III show that a heat-stable toxic substance is produced by *B. cereus* under the same conditions that a similar substance is produced by *B. thuringiensis*. Considering the close relationship of the two organisms (some authors consider them forms of the same species), it would not be surprising if the same substance is being produced by both.

The inoculum obtained from *B. cereus* was less potent than that from *B. thuringiensis*. Perhaps this only reflects a difference in the number of organisms present. But, whatever it is due to, it is interesting to note that the inoculum from *B. cereus* was less potent both from in vivo and from in vitro sources.

Of more biological interest, the fact that the toxin is produced by the non-pathogenic *B. cereus* may imply that the heat-stable toxin has only a minor or even insignificant role in natural infections.

TABLE III

Results of injection of *Galleria* larvae with autoclaved filtrates from in vitro and in vivo cultures of *B. thuringiensis* and *B. cereus*. Values recorded as number of survivors out of 10 larvae injected with each inoculum

Inoculum	Milliliters injected	Inoculum from *B. thuringiensis*	Inoculum from *B. cereus*
Diffusate from liver broth culture	0.01	0	0
	0.001	0	8
	0.0001	10	10
	0.00001	9	10
Sterile liver broth (control)	0.01	10	
Filtrate from infected larvae	0.02	0	1
	0.005	0	10
	0.002	1	10
	0.001	4	8
Filtrate from healthy larvae (control)	0.01	10	

Effect of the Toxin on Galleria Larvae

There is no sudden or dramatic effect following the injection of lethal amounts of the heat-stable toxin. The larvae gradually become lethargic, then moribund, and finally dead. Death takes place 5 to 15 days after injection depending on the amount of inoculum injected. In no case did death occur in less than 4 days even with the most concentrated inocula.

Old larvae may spin a cocoon but it is usually relatively loose and thin-walled. With minimal doses, some larvae not only spin a cocoon but also pupate before succumbing.

Ligated larvae were injected in either the anterior or posterior half. Whichever half was injected displayed the typical symptoms and "died." Apparently the site of action is not an organ or tissue restricted to either the

anterior or posterior half of the animal. A detailed histopathology was not undertaken but it was noticed that some of the larvae receiving heavy doses displayed a random pattern of black spots. These abnormal spots are in the cuticle. They imply a metabolic disturbance but do not identify it.

The injection of a sublethal amount of the toxin into larvae produces no visible effect but does delay emergence of the adults. When the time required for 50% emergence was compared, 80 larvae injected with sublethal but near minimal lethal amounts of the toxin required 30–40% longer than the 80 controls which were injected with either liver broth or distilled water. This shows that there is an effect at sublethal doses rather than a sharp threshold concentration for effect.

The heat-stable toxin must be injected to have any demonstrable effect. The reason for this is not known but feeding tests substantiated it. Following preliminary feeding tests which gave negative results, 20 early instar *Galleria* larvae were confined in petri dishes with a food source containing 16% toxic supernatant by weight. All of the larvae grew to maturity, although 0.001 ml of the supernatant was lethal when injected into large larvae. In a second experiment, varying amounts of the same supernatant were hand-fed to eight American cockroaches. No ill effects were noted in cockroaches ingesting up to 14 times the volume which proved lethal on injection. Finally mosquito larvae (*Aedes aegypti*) were successfully reared in water containing 10% by volume of culture filtrate.

Discussion

No attempt will be made to give a full review of the important work by Hannay, Angus, and Heimpel on the toxicity of *B. thuringiensis*. Suffice it to say that Hannay (4) reported the presence of alkali-soluble, crystalline inclusions in the sporulating cells, and Angus (1) demonstrated that this material was highly toxic. Heimpel (5) produced evidence that a second factor, which he thought to be a lecithinase, is also an active toxic substance. But since both the inclusion bodies and the lecithinase are heat-labile, they cannot represent the toxin with which we have worked. The heat-stable toxin must be a third toxic substance.

Two reasons probably contribute to the fact that the heat-stable toxic principle has not been previously recognized. First, the toxin identified with the inclusion bodies is quite potent. A lethal dose for larvae of *Bombyx mori* is less than 1 μg per gram of larvae (Angus (2)). In contrast, the quantity of culture filtrate required to produce comparable mortality is relatively huge, at least several thousand times as large. Consequently, in experiments involving inclusion bodies, even the controls are likely to receive less than a lethal amount of the heat-stable toxin. Second, a much longer time is required for the heat-stable toxin to produce death. A minimum of 4 days postinjection is required in contrast with 24 hours or less for the heat-labile toxins. It is likely that numerous experiments would be terminated and discarded before the effect of the heat-stable toxin became apparent.

What role this heat-stable toxin may play in the action of *B. thuringiensis* on its host is uncertain. Since it is not toxic following oral administration, and since it (or some similar-acting substance) is also produced by non-pathogenic strains of *B. cereus*, one may well wonder if the heat-stable toxin has any importance in nature. Perhaps its importance is only for laboratory experimentation when injections are being made into the blood stream of insects. In laboratory experiments it can clearly, if unrecognized, lead to unexplained variability and to the death of control animals. In fact, it was the unexpected death of control animals that led to our discovery of this toxin.

But there is existing evidence that in some cases the pathogenicity of some organisms for insects depends on slow-acting substances with at least certain of the characteristics of the heat-stable toxin treated in the present paper. That is, some microorganisms pathogenic for insects kill very slowly, implying a slow-acting toxic material which either is present in small quantity or is of relatively low toxicity. Thus, Beard (3) in a study of the milky disease of Japanese beetle larvae, *Popillia japonica*, reported that the causative agent, *Bacillus popilliae*, is present in the blood of the larvae in such large numbers as to give the larva a typical "milky" appearance. However, despite the enormous numbers of bacteria present, the larvae do not succumb immediately from the disease. In some cases they will even overwinter in the diseased condition. Either *B. popilliae* does not produce as much toxicity as *B. thuringiensis*, or else the beetle larvae are relatively immune to these toxins. The former possibility seems more likely inasmuch as all the insects tested have proved highly susceptible to *B. thuringiensis* when the bacillus is injected into the blood stream.

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ORGANISMS ASSOCIATED WITH SEPTICEMIA IN THE HONEYBEE, APIS MELLIFERA[1]

G. B. LANDERKIN AND H. KATZNELSON

Abstract

Five of seven bacterial cultures received in this laboratory as *Bacillus apisepticus* and *Bacillus sphingidis* and producing a fatal septicemia in honeybees have been shown upon morphological and cultural examination to belong more properly to the family Pseudomonaceae, genus *Pseudomonas*. It is suggested that such organisms now be regarded as *Pseudomonas apiseptica* (Burnside), rather than *Bacillus apisepticus* as originally proposed. The status of the two remaining cultures representing a single *Aeromonas* species requires additional investigation.

Introduction

Bacteria in the blood of adult bees were recorded in 1888 by Cheshire (5); however, a septicemic condition of adult bees was first described in 1928 by Burnside (3). In a subsequent report (4) he gave a complete description of the causal organism and named it *Bacillus apisepticus*. However, it is singular that in no instance was sporulation observed in the original isolates. Preliminary examination of two cultures received in this laboratory as *Bacillus apisepticus* revealed small, motile, Gram-negative, non-sporulating rods, quite obviously not belonging to the genus *Bacillus* as presently construed. Thus, the taxonomic position of this pathogen presented a problem requiring further investigation.

Materials and Methods

A total of eight cultures was received from the Apiculture Division, Experimental Farms Service. These included five strains of *Bacillus apisepticus*, three of which were supplied by Dr. A. S. Michael, Entomological Research Division, U.S. Department of Agriculture, Bethesda, Maryland, and two by the Bacteriology Department of the Ontario Agricultural College, Guelph; the latter two strains originally came from Maryland as did two strains of *Bacillus sphingidis* received at the same time. A single unnamed culture isolated at Guelph and causing a septicemia in bees was also included in this study.

Following preliminary microscopic examination all cultures were plated serially on penassay base agar (Difco) and typical, well-isolated colonies were selected for further study. As far as was possible the procedures outlined in the manual of the Society of American Bacteriologists (12) were followed.

Studies on the pathogenicity of selected strains were carried out by Dr. R. Boch of the Apiculture Division with aerosols prepared in the Bacteriology Division.

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Contribution No. 477 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

Results

Pathogenicity studies revealed that seven of the eight cultures produced a lethal septicemia in bees. The non-pathogenic strain, possibly a predominant contaminant in the original culture, has been tentatively identified as *Micrococcus freudenreichii* and as such is of no further interest in this study.

The remaining seven cultures were readily characterized as belonging to the family Pseudomonaceae and were further separated into two distinct types made up of five similar *Pseudomonas* sp. and two similar *Aeromonas* sp. as described below.

Type 1, Pseudomonas sp., Five Strains

Morphology

Rod forms with rounded ends 0.4–0.6 by 1.0–1.4 μ occurring singly, in pairs, or in short chains; motile, possessing generally one but up to three polar flagella; Gram-negative, endospores not produced.

Cultural Characteristics

Gelatin colonies.—Ivory, opaque, rapid saucer liquefaction.

Gelatin stab.—Rapid napiform to saccate liquefaction, no pigmentation.

Agar colonies.—Circular, smooth, entire, raised, opaque to ivory, certain strains produced a pinkish water-soluble pigment. Diameter after 2 days 34° C, 1.5–3.0 mm.

Agar slant.—Moderate filiform, glistening, opaque to ivory growth, with putrid odor; consistency butyrous.

Nutrient broth.—Moderate to abundant growth, with ring and sediment, certain strains produced a pinkish pigment. Odor putrid.

Litmus milk.—A soft curd is formed within two days, becoming alkaline with slow digestion and no reduction.

Potato slice.—Ivory, grey, smooth, moist, spreading growth, becoming brownish.

Biochemical Characteristics

Acid but no gas is formed from glucose, sucrose, maltose, and mannitol.
Lactose is not fermented.
Nitrates are reduced to nitrites.
Neither indole nor hydrogen sulphide is produced.
Starch is not hydrolyzed.
Porcine blood is not haemolyzed.
Optimum temperature, 34° to 37° C; grows at 10° C and 42° C.
Aerobic facultative, pathogenic for bees.

Type 2, Aeromonas sp., Two Strains

Morphology

Rod forms with rounded ends, 0.4–0.6 by 0.8–1.4 μ, occurring singly or in pairs, motile, possessing generally a single polar flagellum; Gram-negative, endospores not produced.

Cultural Characteristics

Gelatin colonies.—Opaque, circular, convex colonies 1.0 to 1.5 mm in diameter.

Gelatin stab.—Uniform filiform growth, without liquefaction, no pigmentation.

Agar colonies.—Circular, smooth, entire, convex, ivory, 1.5 to 3.0 mm in diameter.

Agar slant.—Moderate, filiform, glistening, ivory, butyrous growth.

Nutrient broth.—Moderate to abundant growth, with ring and sediment. Odor putrid.

Litmus milk.—An acid curd is formed without reduction or digestion.

Potato slice.—Ivory, smooth, moist, glistening growth.

Biochemical Characteristics

Acid and gas are formed from glucose, lactose, sucrose, maltose, and mannitol.

Nitrates are reduced to nitrites.

Neither indole nor hydrogen sulphide is produced.

Starch is not hydrolyzed.

Porcine blood agar plates showed weak haemolysis.

Optimum temperature, 34° to 37° C; grows at 15° C and up to 41° C.

Aerobic facultative, pathogenic for bees.

Discussion

Many references to organisms associated with insect septicemia including members of the genus *Pseudomonas* have been noted by Steinhaus (11), and the recent studies of Bucher and Stephens (2) have demonstrated a laboratory disease of grasshoppers caused by *Pseudomonas aeruginosa.* The causal organisms of hornworm and cutworm septicemias were described by White (13, 14), who named them *Bacillus sphingidis* and *Bacillus noctuarum* respectively. The cultural characteristics of both of these organisms and of *Bacillus apisepticus* as given by Burnside (4) are in close agreement with the five type 1 pseudomonads that were received as three strains of *Bacillus apisepticus* and two strains of *Bacillus sphingidis.* The cultural similarity of *Bacillus sphingidis* and *Bacillus noctuarum* had been previously noted by White (14), who separated them on a serological basis from each other and from *Bacillus acridiorum* (*Coccobacillus acridiorum*) d'Herelle (8), an organism infecting grasshoppers, that was studied systematically by Glaser (6).

That these organisms might be mutant forms of *Pseudomonas aeruginosa* was considered, and all strains were streaked on the pyocyanin enhancement medium A suggested by King, Ward, and Raney (9), with negative results. Two of the type 1 strains did produce a pinkish pigment suggestive of pyorubin (cf. Meader, Robinson, and Leonard (10)); however, its production was not constant. No slime was produced by our cultures in the liquid potassium gluconate medium of Haynes (7), who observed profuse sliminess in all strains

of *Pseudomonas aeruginosa* tested. Similarly only a single type 1 strain oxidized potassium gluconate to 2-ketogluconate, considered by Haynes to be a typical characteristic. It would therefore appear that the five *Pseudomonas* strains are not apyogenic, non-pigmented forms of *Pseudomonas aeruginosa* but types closely related to this organism. Pathogenicity studies with aerosols produced from two known strains of *Pseudomonas aeruginosa* failed to produce septicemia in bees.

The type 2 strains represent a single original isolation by Michael but were received from different laboratories. On the basis of morphology and cultural characteristics they are tentatively considered as belonging to the genus *Aeromonas*. In this genus only four species, none identical with our strains, are described in Bergey's Manual (1). It is suggested that additional isolates of this type be examined before a species name is assigned.

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β-D-1,3 GLUCANASES IN FUNGI[1]

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Abstract

β-D-1,3 Glucanases are of common occurrence in fungi, being detected in the culture filtrates of 96% of the organisms tested in shake flasks and in the sporophores of six basidiomycetes. The enzyme is constitutive. Basidiomycete QM 806 and *Sporotrichum pruinosum* QM 826 are excellent sources of β-D-1,3 glucanase of the exo-type giving glucose as the sole reducing product of laminarin hydrolysis. *Rhizopus arrhizus* QM 1032 produces a β-D-1,3 glucanase of the endo-type giving laminaribiose and higher oligosaccharides as the products of hydrolysis of β-D-1,3 glucans. By controlling the conditions of growth, β-D-1,3 glucanases can be produced free of β-1,4 glucanase (cellulase).

Introduction

β-D-1,3 Glucanases are enzymes hydrolyzing the β-D-1,3-linked polymers of glucose (glucans). Previously they have been known as laminarinases because they were originally tested on laminarin, a polysaccharide from the marine alga, *Laminaria*. Since β-D-1,3 glucans are now known to be of wide occurrence (algae, fungi, higher plants) it seems desirable to give a general name to the enzymes attacking them. A historical review of the nature and occurrence of the glucans is found in the book by Whistler and Smart (16).

The function of β-D-1,3 glucans in plants is not clear. Even less seems to be known of how they are formed or stored. They act as a reserve food in *Laminaria*, and perhaps, also, in sclerotia of the fungus *Poria cocos* (15). In other organisms, they appear to be related to structural materials. The glucan is part of the cell wall in baker's yeast (3). It is found as callose in grape vines (2). In both of the latter, the glucan exists in a somewhat modified form. β-D-1,3-Linked glucose units occur also in polysaccharides of mixed linkage types.

β-D-1,3 Glucanases are ubiquitous enzymes, having been found in fungi (1, 13), bacteria (4), higher plants (16), algae (5), lower forms of sea animals (snails, giant chiton (7)). The greater ease of detection of the enzyme as compared with the substrate perhaps accounts for the wider variety of sources reported for the glucanase. Most reports on the β-D-1,3 glucanases have been incidental to some other problem. Recently (11) a more direct interest has been shown.

In the present report we focus attention on this particular group of enzymes. How frequently do they occur in fungi? How do they hydrolyze their substrata? Can they be obtained free of other polysaccharases? These are some of the questions we hope to answer in part.

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Methods

The organisms were grown on a carbon source in a basal medium containing (per 1000 ml): KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; urea, 0.3 g; $MgSO_4.7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; yeast extract, 0.1 g; and trace elements (Fe, 1.0 μg; Mn, 0.5 μg; Co, 0.5 μg; Zn, 0.8 μg). The cultures (50 ml/250-ml flask) were grown on a reciprocal shaker (90 strokes/minute) at 29° C.

The enzyme-containing solutions were obtained by filtering the cultures through medium porosity, fritted-glass crucibles. Some of the enzyme solutions were concentrated and partially purified by solvent precipitation. Other solutions were dialyzed, concentrated by evaporation (Rotovap), and lyophilized.

The laminarin used in these tests is insoluble laminarin, Batch I.L.31 from the Institute of Seaweed Research, Inveresk, Scotland. It has a moisture content of 11%. The glucose yield on acid and on enzyme hydrolysis indicates that 83% of the dry weight is glucan. The cellulose (Solka Floc) is a highly purified wood product of Brown Co., Berlin, New Hampshire.

β-D-1,3 Glucanase activity was determined as follows: 0.6% laminarin in *M*/20 citrate pH 4.8, 0.5 ml; enzyme solution to be assayed, 0.5 ml; in Folin tubes, 40° C 1 hour.

Reducing sugars produced were determined by the dinitrosalicylic acid method of Sumner and Somers (14). An enzyme solution has one β-D-1,3 glucanase unit (U) per ml if, when assayed as shown, it produces 0.5 mg of reducing sugar as glucose. Cellulase (Cx) was determined by hydrolysis of carboxymethylcellulose as previously described (10).

Paper chromatograms were developed for 16 to 20 hours with isopropanol: acetic acid : water (67:10:23). Reducing sugars were detected with benzidine (6). The movement of compounds is expressed as a comparison with the distance moved by glucose, i.e., $R_G = 1.00$ for glucose.

Enzyme names and abbreviations used herein include:

β-D-1,3 glucanase = laminarinase = polysaccharase acting on long-chain β-D-1,3-linked glucose polymers;

β-D-1,3 oligase = β-D-1,3 glucosidase = β-D-1,3 oligosaccharase acting on short-chain β-D-1,3-linked glucose polymers. This is a somewhat arbitrary separation of enzyme types and may require modification.

Cx = β-1,4 glucanase = cellulase (in part) = polysaccharase acting on long-chain β-1,4-linked glucose polymers.

Results

A. Screening of Fungi for β-D-1,3 Glucanase

One hundred and forty organisms were grown in shake flasks and tested for β-D-1,3 glucanase. All but five produced detectable amounts of the enzyme. The most active cultures are listed in Table I. Penicillia, especially of the *P. luteum* series, appear frequently in this list, but the 12 aspergilli tested produced lower amounts of the enzyme.

TABLE I

Fungi producing high yields of β-D-1,3 glucanase

Organism	QM No.	Carbon source*	β-D-1,3 glucanase, U/ml	Cx = β-1,4 glucanase, U/ml
Alternaria dauci	7170	Cellobiose	7	0
Basidiomycete sp., conidial	806	Cellobiose	160	1.5
Basidiomycete sp., conidial	806	Starch	900	0.2
Basidiomycete sp., conidial	807	Cellobiose	160	1.6
Basidiomycete sp., conidial	592	Cellobiose	10	0.9
Basidiomycete sp., conidial	594	Cellobiose	50	1.3
Basidiomycete sp., conidial	2378	Cellobiose	60	3.2
Humicola fuscoatra	34*e*	Inulin	9	NT†
Myrothecium verrucaria	460	Laminarin	10	0
Paecilomyces varioti	10*a*	Laminarin	8	0
Penicillium echinulo-nalgiovense	7301	Cellulose	13	15.0
Penicillium funiculosum	474	Cellobiose	8	0
Penicillium javanicum	1876	Laminarin	15	0
Penicillium javanicum	6959	Cellulose	24	13.0
Penicillium nigricans-janczewskii (series)	6900	Cellulose	7	16.0
Penicillium pusillum	137*g*	Cellobiose	11	(+)
Penicillium rotundum	1854	Laminarin	24	0
Penicillium spiculisporum	979	Laminarin	38	0
Penicillium stipitatum	6759	Laminarin	10	0
Penicillium vermiculatum	1858	Laminarin	28	0
Penicillium vermiculatum	7316	Cellulose	33	8.0
Penicillium wortmanni	1859	Laminarin	14	0
Pestalotiopsis westerdijkii	381	Cellulose	9	35.0
Poria cocos	7695	Laminarin	40	0.1
Poria monticola	1010	Laminarin	23	0.5
Rhizopus arrhizus	1032	Cellobiose	24	0
Sporotrichum pruinosum	826	Cellulose	600	72.0
Stemphylium sp.	7086	Cellobiose	9	0
Syncephalastrum racemosum	709	Laminarin	18	0
Trichoderma viride	6*a*	Maltose	.6	0

* Carbon source 0.5%; shake flasks.
†NT = no test.

Seven of the fungi were grown on 10 carbon sources (Table II). β-D-1,3 Glucanase was produced in cultures on all substances that supported growth. Yields were no better on laminarin than on other substrates. The enzyme is therefore constitutive in fungi. In this respect, it differs from cellulase and chitinase, which are adaptive (10).

The fruiting bodies of six basidiomycetes were collected in the field and tested for the presence of β-D-1,3 glucanase and other polysaccharases (Table III). β-D-1,3 Glucanase was the dominant polysaccharase in four of these species, one of which, *Polyporus betulinus*, also contained amylase as a major enzyme. Amylase was the dominant polysaccharase present in the sporophore of *Lactarius piperatus*; and cellulase (Cx) in *Clavaria* sp. These two fungi contained little or no β-D-1,3 glucanase. None of the fungi tested had detectable amounts of dextranase (α-D-1,6 glucanase) or polygalacturonase (α-D-1,4 polygalacturonase). Chitinase was present only in trace amounts (18-hour assay). The enzyme values given are based on extracts of fresh sporophore tissue. The amount of β-D-1,3 glucanase is very low compared

TABLE II

Effect of carbon source (0.5%) on β-D-1,3 glucanase production by fungi

Organism	QM No.	β-D-1,3 glucanase U/ml when grown on: Glycerol	Mannitol	Glucose	Xylose	Lactose	Maltose	Cellobiose	Laminarin	Starch	Cellulose
1. *Asp. phoenicis*	1005	0.5	2	5	6	NG†	5	5	5	6	NT†
2. Basidiomycete	806	0.1	2	464	222	NG	395	282	264	416	60
3. *Myrothecium verrucaria*	460	4	1	4	5	0.4	6	7	11	8	2
4. *Paecilomyces varioti*	10*a*	3	4	4	3	NG	3	4	4	4	NG
5. *Rhizopus arrhizus*	1032	1	1	0.8	0.9	NG	1	1.8	1	0.9	NG
6. *Sporotrichum pruinosum**	826	47	13	14	21	NG	15	33	15	19	76
7. *Trichoderma viride*	6*a*	1	1	2	2	4	8	1	4	10	8

* For *S. pruinosum* 0.1% protease peptone was included.
† NT = no test; NG = no growth.

TABLE III

Polysaccharases found in sporophores of basidiomycetes

Sporophores			Enzyme per gram fresh weight			
Fungus	Type	% moisture	β-D-1,3 glucanase, U/g	Amylase, α-1,4 glucanase, U/g	β-1,4 xylanase, U/g	Cx, β-1,4 glucanase, U/g
Lepiota procera	Mushroom	86	14.0	0.4	0.6	0.0
Calvatia cyathiforme	Puffball	NT	9.0	0.2	0.3	0.0
Tremella foliacea	Gelatinous	95	1.0	0.0	0.0	0.0
Polyporus betulinus	Bracket	82	9.0	1.5	0.1	0.3
Lactarius piperatus	Mushroom	NT	0.3	6.0	0.0	0.0
Clavaria sp.	Coral	90	0.6	0.0	0.6	16.0

with the amounts produced by fungi growing in shake flasks (Table I). An "average" fungus in shake culture produces about 100 times as much enzyme per unit weight.

Of the commercial enzyme preparations we have examined, only the following have appreciable β-D-1,3 glucanase activity: cellulase (Takamine), 3.1 U/mg; hemicellulase (Nutr. Biochem. Co.), 1.2 U/mg; enzyme 19AP (Röhm and Haas), 0.9 U/mg; and lipase (Gen. Biochem. Co.), 0.4 U/mg. Traces of activity only were found in mylase P (NBC), pectinol 10 M (R and H), diastase, malt (GBC), ficin (Merck), pectinase (NBC), and takadiastase (Parke Davis Co.). No activity could be detected in: pancreatin (NBC), steapsin (NBC), glucose oxidase (Takamine), lactase (NBC), trypsin (Eimer and Amend), invertase (NBC), pepsin (Merck). These data indicate that commercial sources contain relatively little β-D-1,3 glucanase (compare with Table IV).

Three of the active fungi (Table II) were selected for further study: Basidiomycete QM 806, *Sporotrichum pruinosum* QM 826, and *Rhizopus arrhizus* QM 1032.

1. Basidiomycete QM 806

This fungus is the best producer of β-D-1,3 glucanase that we have found. It grows well on a variety of carbon sources (Table II), and (fortunately) can produce β-D-1,3 glucanase in the absence of cellulase (β-1,4 glucanase). Starch (1%), used for the routine production of the enzyme, is rapidly digested (4–7 days), during which time the pH falls from 6.2 to 3.0 and then rises again to 6.5. The β-D-1,3 glucanase levels increase rapidly after the starch has been consumed, reach a maximum at 10–14 days, and then decrease.

The highest yields on starch have been 900 β-D-1,3 glucanase units per ml of culture medium. In these solutions the Cx is barely perceptible (0.1–0.2 U/ml). On the other hand, when grown on cellulose, this organism produces high yields of cellulase (about 100 Cx U/ml), with relatively low yields of β-D-1,3 glucanase (40–60 U/ml).

Basidiomycete QM 806 has been submitted to taxonomists who specialize in identification of basidiomycetes in culture. Unfortunately, it is an unfamiliar

species and remains unidentified. It produces conidia in culture and has clamp connections. It has been suggested that we place it in the form genus *Ptychogaster*. Our QM culture collection has several similar isolates, apparently the same fungus, all originating in tropical areas. All are cellulolytic, produce very good yields of β-D-1,3 glucanase, and behave alike in culture. Known species of basidiomycetes have been tested for comparison, but produced low levels of β-D-1,3 glucanase: *Ptychogaster rubescens* (QM 1011), *Schizophyllum commune* (QM 812), *Coprinus sclerotigenus* (QM 933), and *Polyporus versicolor* (QM 1013). *Poria cocos* (QM 7695, QM 7696) and *Poria monticola* (QM 1010) produced yields of enzyme only a little lower than those obtained from some members of the Basidiomycete 806 complex (see Table I).

2. Sporotrichum pruinosum QM 826

This organism was selected for study because of the high β-D-1,3 glucanase yields (approaching those of QM 806), and because it simultaneously produces very high yields of cellulase (Cx) when grown on cellulose. When grown on other substrates it still produces good β-D-1,3 glucanase yields, but little or no cellulase.

Cellulose is the best of the tested carbon sources for β-D-1,3 glucanase (and Cx) production by *Sporotrichum pruinosum* QM 826 (Table II). Cellulase develops earlier than β-D-1,3 glucanase (Fig. 1A). In this respect β-D-1,3 glucanase resembles amylase in its development in cellulolytic cultures (10). The yields of enzyme are a function of the substrate concentration (Fig. 1B), highest yields being obtained at the highest concentration tested (2%). Unfortunately, the incubation time to reach maximum values also increases.

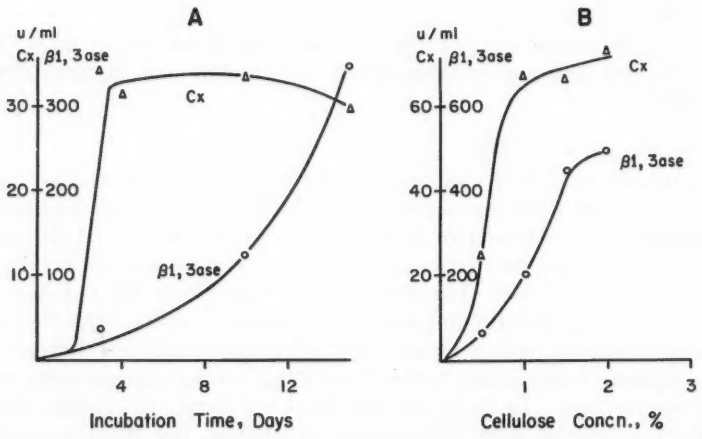

FIG. 1. Production of β-D-1,3 glucanase, and of Cx, by *Sporotrichum pruinosum* QM 826. A. Development of activity during incubation. Substrate = 0.5% Solka Floc (cellulose) + 0.1% proteose peptone. B. Effect of cellulose concentration on yields. Medium includes 0.1% proteose peptone. Experiment lasted 32 days.
△ — △ Cx, ○——○ β-D-1,3 glucanase.

For a 2-week incubation period, 1.5% cellulose is optimal. Addition of proteose peptone (0.1%) increases the cellulase (Cx) while decreasing the β-D-1,3 glucanase yields of this organism.

3. Rhizopus arrhizus QM 1032

This organism produces much less β-D-1,3 glucanase than do Basidiomycete QM 806 and *Sporotrichum pruinosum*. Its interest lies in the difference between its glucanase and that of the other two fungi, and in the relative absence of a β-D-1,3 oligase, features which make it possible to accumulate intermediates in the hydrolysis of β-D-1,3 glucans.

The low yields (about 1 U/ml) obtained on the basal medium (Table II) were improved markedly by the addition of 0.1% proteose peptone. On 0.6% cellobiose yields of 24 U/ml were obtained (vs. 1.0 U/ml on cellobiose in absence of peptone). (In our assay all reducing sugar produced is determined as glucose. Since *Rhizopus arrhizus* produces mostly dimer and trimer, the unit value is not strictly comparable to the unit obtained with organisms producing largely glucose.) The yields on glucose and on glycerol were about half those on cellobiose. No cellulase was found in any of these filtrates.

B. Concentration and Purification of β-D-1,3 Glucanase

The enzymes of the culture solutions were precipitated by the addition of two volumes of cold acetone. The precipitates were fractionated to some extent by dissolving and reprecipitating with increasing concentrations of ethyl alcohol. In *Sporotrichum pruinosum*, most of the β-D-1,3 glucanase (80%) was soluble in 50% alcohol, but insoluble in 75% alcohol. Some of the enzyme preparations are listed in Table IV, with data also for cellulase activities.

TABLE IV

A comparison of the best enzyme preparations

Source of enzyme	Grown on:	β-D-1,3 glucanase, U/mg	Cx, U/mg
Basidiomycete QM 806	Starch	352.0	0.2
Basidiomycete QM 806	Cellulose	17.0	137.0
Sporotrichum pruinosum QM 826	Cellulose	370.0	37.0
Rhizopus arrhizus QM 1032	Cellobiose	40.0	0.0

C. Properties of the β-D-1,3 Glucanases of Basidiomycete QM 806, Sporotrichum pruinosum QM 826, Rhizopus arrhizus QM 1032

1. Enzyme Activity

pH.—The pH optimum for β-D-1,3 glucanase of all three fungi is about 4.5 in citrate buffer. There is very little activity above pH 7.0 or below pH 3.0. The *Rhizopus* enzyme is inactive below pH 4.0.

Temperature.—Under the conditions of the assay (pH 4.8, 1 hour, laminarin 3 mg/ml), maximum activity takes place at 60° C (Fig. 2B, for QM 806).

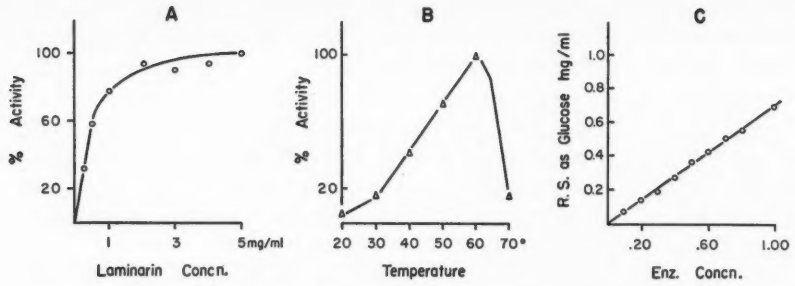

FIG. 2. β-D-1,3 Glucanase of Basidiomycete QM 806. A. Effect of substrate concentration, in reaction mixture (time, 30 minutes). B. Effect of temperature (pH, 4.7; time, 60 minutes). C. Effect of enzyme concentration (time, 30 minutes).

Inactivation is rapid at 70° C. The *Rhizopus* enzyme is slightly less heat stable than the others. At pH levels other than the optimum, inactivation is much more rapid. At pH 6.5, for instance, 90% of the enzyme is inactivated in 30 minutes at 50° C.

Substrate concentration.—The maximum rate of activity is reached at substrate concentrations of 2 mg/ml of reaction mixture (Fig. 2A). Below this value, the rate falls off sharply.

Enzyme concentration, and time of incubation.—Under the conditions of the assay, straight line relationships hold for sugar production vs. enzyme concentration (Fig. 2C), and for sugar production vs. time of incubation. Deviation from this occurs when the amount of reducing sugar (as glucose) exceeds 0.90 mg/ml.

Substrate specificity.—Our β-D-1,3 glucanase preparations are contaminated with other enzymes, and as a result we have not yet thoroughly investigated substrate specificity. We have tried pachyman (from sclerotia of *Poria cocos*) and found that the enzyme hydrolysis products (glucose, laminaribiose, laminaritriose) correspond with those obtained from laminarin. This supports the earlier identification of pachyman as a predominantly β-D-1,3-linked glucan (15).

2. Hydrolysis Products of Laminarin-Using Enzymes

Laminarin has been hydrolyzed by our β-D-1,3 glucanase preparations (Table V).

The low value for *R. arrhizus* is due to the fact that the reaction was not continued to the time of glucose appearance. The values for hydrolysis by Basidiomycete QM 806 and by *Sporotrichum pruinosum* enzymes agree with the value for acid hydrolysis.

Chromatograms of the hydrolyzates of laminarin (Fig. 3) indicate that glucose is produced directly by the action of β-D-1,3 glucanases of Basidiomycete QM 806, and of *Sporotrichum* (latter not shown). At no time during the hydrolysis is there more than a trace of intermediate (at most, a trace of

TABLE V

Enzyme hydrolysis products of laminarin

Preparation from:	Extent of hydrolysis*	Products
Basidiomycete QM 806	83%	Glucose
Sporotrichum pruinosum QM 826	83%	Glucose
Rhizopus arrhizus QM 1032	43%	Dimer; trimer

* From reducing value calculated as glucose.

dimer). In contrast to this is the hydrolysis by *Rhizopus*. Here dimer (R_G 0.69) and trimer (R_G 0.33) are produced quickly, but glucose appears only after long incubation.

The immediate production of glucose from laminarin by the preparation from Basidiomycete QM 806 might be due to the presence of an oligase acting on intermediates. To test this we measured oligase activity using as substrate the dimer–trimer mixture produced by *Rhizopus* enzyme acting on laminarin. As a measure of oligase activity we used the time required to produce 1 mg of glucose (determined with glucose oxidase). There is very low oligase activity compared with the amount of β-D-1,3 glucanase activity in preparations of any of these organisms (Table VI). There is enough, however, that on long hydrolysis and with strong enzyme solutions both intermediates are hydrolyzed to glucose (Fig. 3 shows this for *Rhizopus*).

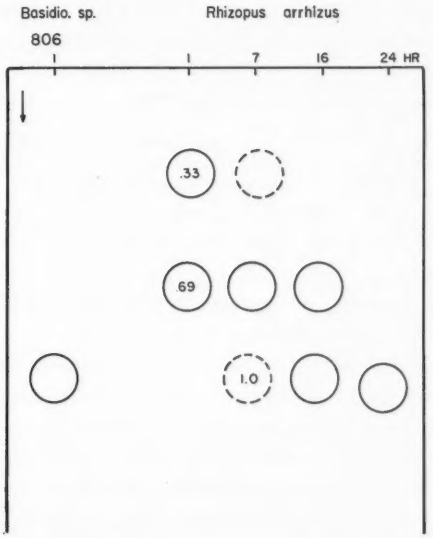

FIG. 3. Chromatograms of enzyme hydrolyzates of laminarin. 806 = β-D-1,3 glucanase of Basidiomycete QM 806. Numbers in spots = R_G values, i.e., the distance moved relative to that moved by glucose. Broken circles indicate fainter spots.

TABLE VI

Time required to produce 1 mg of glucose per ml by 1 unit of β-D-1,3 glucanase

Enzyme of:	Time, hours*	
	From laminarin	From dimer–trimer
Basidiomycete QM 806	2.5	240
Sporotrichum pruinosum QM 826	2.0	68
Rhizopus arrhizus QM 1032	70.0	72

* Reaction mixture: substrate 3 mg/ml, pH 4.8, 40° C, 1 unit β-glucanase.

The trimer disappears much more rapidly than the dimer. The basidiomycete filtrate produces glucose 100 times as fast from laminarin as from an equal concentration of dimer–trimer mixture; the *Sporotrichum* filtrate, 30 times as fast. Thus, the absence of dimer and trimer in hydrolyzates of laminarin by these filtrates is not due to an active oligase, but must be due to direct production of glucose from laminarin. On the other hand, the *Rhizopus* filtrate produces glucose at about the same rate from laminarin and from dimer–trimer mixture, the hydrolysis of the latter being the limiting factor.

The diverse nature of the glucanase systems of Basidiomycete QM 806 and of *Rhizopus* was brought out in another type of experiment (Fig. 4). The glucanase of Basidiomycete QM 806 was added to the *Rhizopus*–laminarin hydrolyzates at various times. In similar enzyme systems, a doubling of enzyme concentration usually gives an increased over-all reaction. But here the action of the glucanase of Basidiomycete QM 806 was markedly inhibited

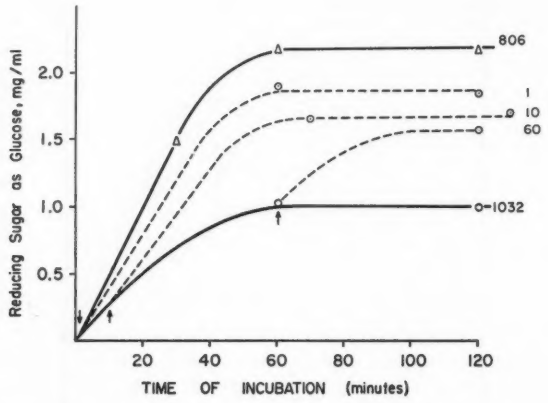

FIG. 4. Inhibition of activity of one β-D-1,3 glucanase (806) by the prior action of another (1032) on laminarin. 806 = glucanase of QM 806 alone; 1032 = glucanase of QM 1032 alone; 1, 10, 60 = glucanase of QM 1032 acting alone for 1, 10, 60 minutes, at which time (↑) the glucanase of QM 806 was added. These hydrolyzates have a full complement of both enzymes.

by prior action of the *Rhizopus* glucanase for as little time as 1 minute. Apparently, the action of the *Rhizopus* enzyme produces short chains which are resistant to attack by the Basidiomycete QM 806 enzyme.

Discussion

A. Occurrence and Production of β-D-1,3 Glucanase

β-D-1,3 Glucanases are present in most fungi. They are secreted into the medium where they function as digestive enzymes hydrolyzing glucans produced by other organisms. Extracellular digestive enzymes (cellulase, chitinase, xylanase) are usually adaptive in fungi. The β-D-1,3 glucanases are constitutive. They may also function in another capacity, one common to all fungi, the intracellular hydrolysis (and synthesis) of a reserve material containing β-D-1,3 glucosidic linkages. As such, these enzymes resemble amylase which is also constitutive in many fungi, which may function intra- and extra-cellularly, and which appears later in the growth cycle than cellulase and other adaptive enzymes. Glycogen, a substrate for amylase, is well known as a reserve food in fungi, but β-D-1,3 glucans have been reported in only three fungi: baker's yeast, *Poria cocos*, and perhaps *Sclerotinia libertiana* (8). *Poria cocos* is the only fungus we have tested which is known to produce both the substrate β-D-1,3 glucan and the enzyme β-D-1,3 glucanase. We expect that further investigations will show that β-D-1,3 glucans are of rather widespread occurrence in fungi.

Although nearly all fungi produce β-D-1,3 glucanase, there are great differences in the amounts produced by the various organisms. *Myrothecium verrucaria* and *Aspergillus niger*, organisms which have been used as sources of β-D-1,3 glucanase in the works of others, (1, 13) are not very good producers of this enzyme. Likewise, most commercially available enzyme preparations contain little of this polysaccharase. Yet β-D-1,3 glucanase is about as common in fungal enzyme preparations as is amylase. This contamination of enzyme preparations should be considered in reporting on the specificity of enzymes for any particular linkage, e.g., the ability of cellulase to hydrolyze polysaccharides containing linkages other than β-1,4 (9, 12).

The organisms which we have studied are excellent sources of β-D-1,3 glucanase. The yields obtained are higher than those from other known sources by a factor of 10–100. It is interesting that the production of a constitutive enzyme should be influenced greatly by the carbon source, and that the carbon source best for each organism should be different. Thus, growth on soluble starch leads to the greatest production of β-D-1,3 glucanase in Basidiomycete QM 806 whereas cellulose gives the lowest yields. On the other hand, cellulose is the best carbon source for *Sporotrichum pruinosum*, and cellobiose seems to be a superior carbon source for the production of the enzyme by *Rhizopus arrhizus*. Growth conditions may be affecting the build-up of a reserve β-D-1,3 glucan, and this in turn may determine the level of enzyme produced.

B. Mode of Action

β-D-1,3 Glucanases are of two types (11) which we might designate (after Duncan *et al.* (5)):

(*a*) The endo- or random-splitting type. Hydrolysis of β-D-1,3 glucans by this enzyme yields laminaribiose and higher oligosaccharides. It is found in wheat, barley, rye, marine algae, and in *Rhizopus arrhizus*.

(*b*) The exo- or endwise-splitting type. Hydrolysis by this enzyme produces glucose as the initial and sole product. Found in almond emulsin and in fungi (Basidiomycete QM 806, *Sporotrichum pruinosum*, etc.).

This classification is based on the products of hydrolysis, but like all such schemes, it may go beyond the facts. The action of the exo-type enzymes implies the removal of a single glucose from chain A, followed by removal of a unit from chain B, etc., leading eventually to what we find to be a rather resistant dimer. Assuming a degree of polymerization of 20 for laminarin, the dimer would amount to about 10% of the product and should be readily detectable by our techniques. Its apparent absence suggests the possibility of an "unzippering" action whereby each molecule of laminarin is completely hydrolyzed to glucose before the enzyme moves on to the next molecule of substrate. More data are required to resolve this problem.

Investigations by others indicate that the β-D-1,3 glucanases may be a family of enzymes, each fungus producing one or more members of the family. In *Aspergillus niger* three β-D-1,3 components were found and these differed from the three β-1,4 glucanases (= cellulases (13)). Other workers have investigated the possibility that phosphorolysis might be involved but only negative results (5) were reported.

What part, if any, does a β-D-1,3 oligase (glucosidase) play in the hydrolysis of a β-glucan? By analogy with other polysaccharide-splitting systems, there should be a role for an oligase. Certainly the endo-β-D-1,3 glucanase should be complemented by an enzyme whose function it is to hydrolyze the dimer and trimer. For the exo-β-D-1,3 glucanase systems, there seems to be no requirement for an oligase, glucose being the immediate product of the reaction.

Acknowledgments

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A METHANOL-UTILIZING BACTERIUM

II. STUDIES ON THE PATHWAY OF METHANOL ASSIMILATION[1]

T. KANEDA[2] AND J. M. ROXBURGH

Abstract

Two simultaneous pathways for the fixation of carbon from one-carbon compounds by a *Pseudomonas* sp. (PRL-W4) are postulated. Carbonate and formate form parts of one pathway and methanol and formaldehyde the other. It is presumed that both the formate and formaldehyde are incorporated as serine by the organism. The energy necessary for the incorporation of carbonate carbon can be supplied by the oxidation of methanol or of formate but not formaldehyde. No formaldehyde dehydrogenase activity was found.

Introduction

In the first paper in this series (6) a *Pseudomonas* sp., PRL-W4, capable of utilizing methanol as sole carbon source and requiring biotin for growth was described. The mechanism involved in the assimilation of carbon from methanol into the cell is of interest, particularly as the organism shows some of the characteristics of an autotroph in the sense of utilizing carbon from carbon dioxide. Substrates labelled with C^{14} were used to obtain some indication of the pathways of carbon assimilation and the results are discussed in this paper.

Materials and Methods

Culture

The organism was maintained and grown as described previously (6). In experiments with growing cells the radioactive compounds were not sterlized, but they apparently did not introduce any detectable contamination.

For the preparation of the sonic extract a large batch of cells was prepared in a 100-gal fermentor. After incubation for 3 days at 30° C about 350 g of wet cells were recovered by passing the broth through a Sharples centrifuge. The cells were suspended in 800 ml of $M/100$ phosphate buffer (pH 7.8) and 8 mg of reduced glutathione in 40 ml of molar phosphate buffer (pH 7.8) added. The suspension was subjected to the full intensity of a 10-kc Raytheon sonic oscillator for 10 minutes and the debris removed by centrifuging. To the supernatant was added 40 ml of 2% protamine solution and 80 ml of a saturated solution of ammonium sulphate (adjusted to pH 7.8). After 15 minutes the precipitate was removed by centrifugation and the clarified supernatant dialyzed against $M/100$ phosphate buffer (pH 7.8) for 18 hours.

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Preparation of Samples and Measurement of Radioactivities

Two methods of measuring the activity of samples were employed. Where the specific activity of the carbon in a particular sample was required, the sample was oxidized to carbon dioxide and the activity determined in a gas counter. Where only the total or relative activity of a solid sample was required, the sample was dried on a sample pan in a desiccator over calcium chloride or in an oven at 55° C and the activity determined using a gas flow counter.

Where the specific activities of the individual one-carbon compounds were to be determined, the scheme of separation shown schematically in Fig. 1 was employed. Growth was stopped by the addition of 1 to 2 ml of 9 *N* sulphuric acid. The carbon dioxide evolved was trapped in 0.33 *N* sodium hydroxide and precipitated as barium carbonate. The cells were recovered by centrifugation, washed with 0.5% sodium chloride solution, and then dried. The dimedone derivative of formaldehyde was precipitated from the neutralized supernatant and washings, filtered, and dried. Methanol was distilled from the slightly basic filtrate and oxidized to carbon dioxide using ceric ammonium nitrate followed by mercuric oxide (3, 10). The carbon dioxide was again absorbed in sodium hydroxide solution, precipitated as barium carbonate, and dried. The residue from the methanol distillation was acidified and the formic acid in turn recovered by distillation. The distillate was oxidized using mercuric oxide and the carbon dioxide formed was recovered as barium carbonate as described above.

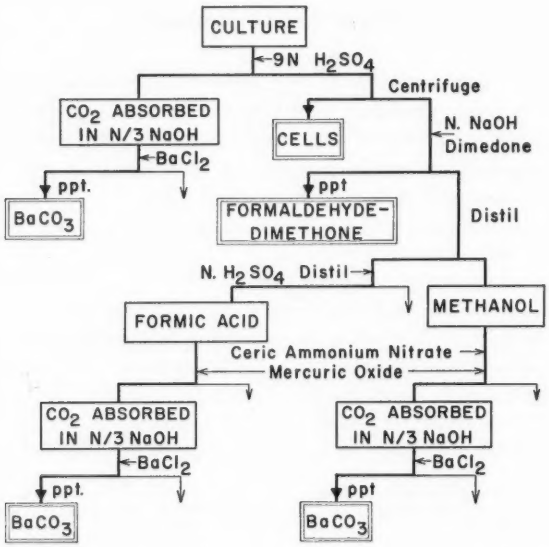


FIG. 1. Method of separation of one-carbon compounds and cells for determination of specific activities.

This method of fractionation gives samples containing the carbon from carbonate, formate, and methanol as barium carbonate, the carbon from formaldehyde as the dimedone derivative and the cells as such. These dried solid samples were oxidized to carbon dioxide for counting by wet combustion.

In some experiments in which resting cells were used only the activity of the cells was measured. The reaction mixture (0.5 ml) with the exception of the labelled compound was placed in a small test tube (12×150 mm). The labelled compound was added and after a specific time, usually 30 minutes, the reaction was stopped by the addition of 0.25 ml of 4 *N* hydrochloric acid. A 0.5 ml sample of the acidified mixture was placed on a plastic sample pan and dried at 55° C. Under the conditions used all the methanol, formaldehyde, formic acid, and carbon dioxide escape and the activity remaining represents carbon fixed by the cells.

Experimental and Results

Only limited information could be obtained from experiments using growing cells, since low levels of carbonate and of formate inhibited growth of PRL-W4 and formaldehyde stopped growth entirely. However cultures were grown on media containing labelled methanol, with and without added unlabelled carbonate or formate, and on media containing unlabelled methanol with labelled carbonate or formate added. After 6 days the specific activity of the cell carbon was determined and compared with the specific activity of the carbonate in the same experiment. In all cases but one the specific activities were found to be nearly identical (Table I).

Resting cell experiments using labelled methanol showed that carbonate, formate, and formaldehyde did not affect the respiration of the cells but that the amount of carbon fixed from the methanol was reduced by the

TABLE I

Specific activity of carbon from cells and from carbon dioxide in cultures of PRL-W4

Substrate carbon source	Cell yield, mg	Specific activity, mμc/mM of carbon	
		Cells	Carbon dioxide
Methanol-C^{14}*	4.2	426	457
+ formate	3.8	429	414
Methanol-C^{14}*	5.9	461	484
+ carbonate	6.7	475	459
Formate-C^{14}†	11.2	25.7	22.4
+ methanol	5.0	30.1	32.0
Carbonate-C^{14}‡	3.1	2980	1393
+ methanol	6.0	1446	1403
Methanol-C^{14}§	2.3	48.6	56.0

NOTE: Medium: Inorganic salts, methanol 0.125 *M*, biotin 1 μg per liter, carbonate 0.008 *M*, and formate 0.0125 *M* as indicated; 40 ml in 250-ml flask.
Culture: Six days on rotary shaker at 30° C.
*504 mμc/mM.
†83.9 mμc/mM.
‡15,200 mμc/mM.
§50.4 mμc/mM.

TABLE II

Respiration and carbon fixation by resting cells of PRL-W4

Substrate carbon source			Total activity of cells, c.p.m.	Respiration rate, μM O_2/hour
Compounds	Concentration, molar	Specific activity, mμc/mM		
Methanol-C^{14}	.01	121	2178	5.64
Methanol-C^{14}	.01	121	697	6.08
+ carbonate	.01	0		
Methanol-C^{14}	.01	121	662	6.72
+ formate	.01	0		
Methanol-C^{14}	.01	121	252	5.52
+ formaldehyde	.01	0		
None (endogenous rate)			—	2.94
Methanol-C^{14}	.01	121	1263	5.98
+ cyanide	10^{-5}	0		
Methanol-C^{14}	.01	121	87	2.28
+ cyanide	10^{-4}	0		
Methanol-C^{14}	.01	121	20	0.14
+ cyanide	10^{-3}	0		
Methanol-C^{14}*	.01	121	427	7.64
Methanol-C^{14}*	.01	121	470	7.90
+ thiosemicarbazide	.02	0		

NOTE: Conditions: 0.1 ml of cell suspension, phosphate buffer pH 7.8, total volume 0.5 ml, at 30° C for 30 minutes.
*A different cell preparation used.

addition of any of these compounds (Table II). The reduction was especially marked when formaldehyde was added. Under the same conditions the fixation of carbon from methanol was reduced to a greater extent than the respiration rate by low concentrations of cyanide (Table II). Thiosemicarbazide, another aldehyde reagent, at a much higher concentration, did not inhibit either carbon fixation or respiration.

The sonic extract from cells of PRL-W4 fixed carbon from labelled methanol, but this fixation was not inhibited by carbonate (Table III). In addition, the whole cells were capable of fixing carbon from labelled carbonate in the presence of unlabelled methanol but the cell extract was not. Very little fixation was obtained with either cells or cell extract from a substrate containing only carbonate as a source of carbon.

At higher substrate concentrations the individual one-carbon compounds were recovered and their activities determined (Table IV). The addition of formaldehyde and carbonate inhibited the fixation of carbon from labelled methanol. A certain amount of activity was found in the formaldehyde recovered but very little in the carbonate. Formate and carbonate did not inhibit fixation of methanol carbon and considerable activity was present in the recovered formate. Again the carbonate had little activity. Carbonate alone at these higher concentrations did not inhibit fixation and the recovered carbonate was heavily labelled.

TABLE III

Fixation of carbon by resting cells and cell extract of PRL-W4

Compounds	Concentration, molar	Specific activity, mμc/mM	Total activity fixed, c.p.m.*	
			By cells	By cell extract
Methanol-C^{14}	.01	121	996	259
Methanol-C^{14}	.01	121	451	267
+ carbonate	.01	0		
Methanol	.01	0	483	11
+ carbonate-C^{14}	.01	426		
Carbonate-C^{14}	.01	426	147	22

NOTE: Conditions: 0.5 ml total volume containing either .05 ml bacterial suspension or 0.20 ml sonic extract from cells. Also phosphate buffer, pH 7.8. At 30° C for 30 minutes.
*Samples dried at 55° C. Methanol, formate, formaldehyde, and carbonate are removed by this treatment.

TABLE IV

Resting cell cultures of PRL-W4 incubated with labelled methanol

Substrate carbon source			Specific activity, mμc/mM of carbon		Total activity, c.p.m.		
Compounds	Concentration, molar	Specific activity, mμc/mM	From cells	Methanol	Formaldehyde	Formate	Carbonate
Methanol-C^{14}	.030	120	0.16	76.9	0.49	0.35	1.13
Methanol-C^{14}	.030	120	0.05	105.3	0.55	0.27	0.05
Formaldehyde	.023	0					
Carbonate	.030	0					
Methanol-C^{14}	0.030	120	0.21	82.0	0.21	2.52	0.34
Formate	0.030	0					
Carbonate	0.030	0					
Methanol-C^{14}	0.030	120	0.31	79.8	0.65	0.70	1.30
Carbonate	0.030	0					

NOTE: Conditions: 10 ml total volume containing bacterial suspension equivalent to 24.5 mg dry cell weight. At 30° C for 30 minutes in pH 7.8 phosphate buffer.

TABLE V

Resting cell cultures of PRL-W4 incubated with labelled carbonate

Substrate carbon source			Specific activity, mμc/mM of carbon				
Compounds	Concentration, molar	Specific activity, mμc/mM	From cells	Methanol	Formaldehyde	Formate	Carbonate
Carbonate-C^{14}	.033	10,700	0.25	—	—	—	9,620
Carbonate-C^{14}	.033	10,700	7.12	21.0	—	—	9,330
+ methanol	.033	0					
Carbonate-C^{14}	.033	10,700	0.05	26.8	—	—	10,180
+ formaldehyde	.014	0					
Carbonate-C^{14}	.033	10,700	3.03	18.1	0	1.99	9,820
+ formate	.033	0					

NOTE: Conditions: 30 ml total volume containing bacterial suspension equivalent to 30.1 mg dry cell weight. At 30° C for 30 minutes in pH 7.8 phosphate buffer.

Resting cell experiments using labelled carbonate showed that carbon was fixed from carbonate when formate or methanol was added but not when formaldehyde was added or when carbonate supplied the sole carbon source (Table V). Methanol stimulated the fixation of carbon from carbonate to a greater extent than formate.

Labelled formaldehyde was incorporated by resting cells to a lesser extent when unlabelled formate or carbonate was added (Table VI) but added methanol had little effect. The amount of activity in the carbonate recovered was high when formaldehyde alone was used, considerably less when carbonate or formate was added, and low when methanol was present. Cells incubated with labelled methanol fixed much more activity than cells incubated with labelled formaldehyde under the same conditions (Table VI).

TABLE VI

Resting cell cultures of PRL-W4 incubated with labelled formaldehyde

Substrate carbon source			Specific activity, mμc/mM of carbon		Total activity in carbonate, mμc
Compounds	Concentration, molar	Specific activity, mμc/mM	From cells	Carbonate	
Formaldehyde-C^{14}	0.0167	20,000	5.57	—	35.0
Formaldehyde-C^{14}	0.0167	20,000	4.01	—	4.45
+ methanol	0.33	0			
Formaldehyde-C^{14}	0.0167	20,000	1.48	—	11.52
+ formate	0.33	0			
Formaldehyde-C^{14}	0.0167	20,000	1.68	15.6	12.8
+ carbonate	0.33	0			
Methanol-C^{14}	0.033	10,000	10.32	—	6.52

NOTE: Conditions: 30 ml total volume containing bacterial suspension equivalent to 28.3 mg dry cell weight. At 30° C for 1 hour in pH 7.8 phosphate buffer.

TABLE VII

Effect of serine and glycine on the assimilation of labelled methanol by sonic extract of cells of PRL-W4

Substrate carbon source			Total activity, c.p.m.*	
Compounds	Concentration, molar	Specific activity, μc/mM	Whole culture	Supernatant†
Methanol-C^{14}	.004	75	450	190
Methanol-C^{14}	.004	75	410	240
+ glycine	.020	0		
Methanol-C^{14}	.004	75	490	220
+ serine	.020	0		
Methanol-C^{14}	.004	75		
+ glycine	.020	0	730	210
+ serine	.020	0		

NOTE: Conditions: 0.5 ml total volume containing sonic extract equivalent to 16 mg dry weight. At 30° C for 30 minutes in pH 7.8 phosphate buffer.
*Samples dried at 55° C. Methanol, formate, formaldehyde, and carbonate are removed by this treatment.
†Centrifuged at 2500 × g.

The effect of serine and glycine on the fixation of carbon from methanol by the sonic extract of cells of PRL-W4 was tested, since serine has been shown to be an early intermediate in the metabolism of methanol by this organism (7). Neither glycine nor serine alone stimulated the fixation of carbon, but the two together had a considerable effect (Table VII).

Finally, experiments with the sonic extract and diphosphopyridine nucleotide (DPN) were conducted for the determination of specific dehydrogenases. The results shown in Fig. 2 indicate that formate and methanol dehydrogenases are definitely present but there is no evidence of the presence of a formaldehyde dehydrogenase.

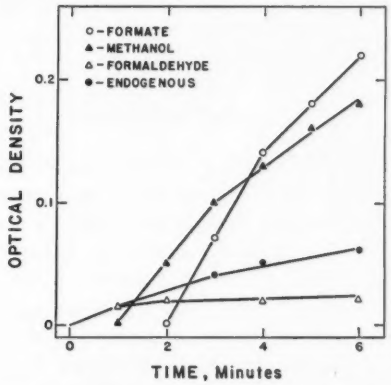


FIG. 2. Determination of DPN-linked dehydrogenase activity in cell-free extract of cells of PRL-W4. Optical density measured at 340 mμ. Spectrophotometer cells contained sonic extract (including reduced glutathione) DPN and the indicated substrate. The curve labelled "Endogenous" was obtained using a cell containing DPN and the sonic extract only.

Discussion

The results obtained in this series of experiments can be summarized briefly as follows:

(*a*) In actively growing cultures the specific activity of cell carbon and carbonate carbon are roughly the same.

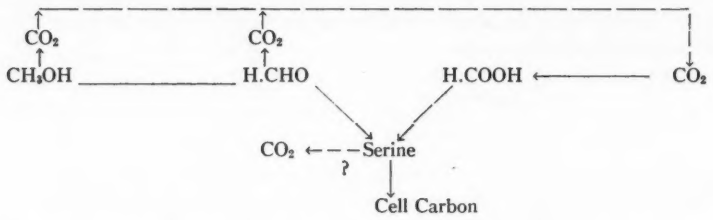
(*b*) Formaldehyde inhibits the fixation of carbon from methanol very strongly, formate and carbonate less so. Formate and carbonate accumulate activity from the methanol to a considerable extent, formaldehyde rather less.

(*c*) Carbonate is utilized very slowly by resting cells in the absence of an energy source. Methanol and formate can provide this energy but formaldehyde cannot.

(*d*) Methanol and formaldehyde dehydrogenases were shown to be present but formaldehyde dehydrogenase activity was not found.

(*e*) Methanol carbon fixation by a cell-free extract was greater in the presence of both serine and glycine than in the absence of one or both.

It seems probable from these results that methanol and formaldehyde form parts of one pathway by which carbon is fixed and that formate and carbonate form parts of another. Since mechanisms for the oxidation of either methanol or formaldehyde or both to carbon dioxide are present the two systems are linked through carbonate. Both pathways presumably involve serine as an intermediate (7). The scheme shown in outline below satisfies all the evidence accumulated to date.



The energy required for the fixation of carbon dioxide by way of the formate pathway can be supplied by oxidation of either methanol or formate but not formaldehyde. The carbon dioxide produced by the oxidation of methanol and formaldehyde is partially incorporated by way of formate so that both schemes are active when methanol is present in the medium.

The absence of a formaldehyde dehydrogenase system is by no means certain but seems probable from the evidence. Both Strittmatter and Ball (11) and Huennekens, Hatefi, and Kay (4) found that this dehydrogenase reduced DPN, although in the latter case TPN stimulated the enzymatic reaction markedly.

The mechanism of serine synthesis from glycine and either formaldehyde or formate has been the subject of several investigations (1, 2, 8, 9, 12). Tetrahydrofolic acid (or tetrahydropteroylglutamic acid) is recognized as an essential cofactor (1, 8, 12). If this is the mechanism operative in cultures of PRL-W4, both serine and glycine would be expected to have some effect on the fixation of carbon from methanol by the sonic extract. The lack of effect except when both serine and glycine are present leaves this point in doubt. The phosphoserine route investigated by Ichihara and Greenberg (5) is an alternate possibility.

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A RELATIONSHIP BETWEEN THE FREE AMINO ACID POOL, DIPICOLINIC ACID, AND CALCIUM FROM RESTING SPORES OF BACILLUS MEGATERIUM[1]

I. ELIZABETH YOUNG[2]

Abstract

All of the free amino acids and calcium dipicolinate are released from disrupted spores of *Bacillus megaterium* to a hot water extract. Chromatographic resolution of the extract reveals six free amino acid spots and the dipicolinate running as a two-limbed band through the chromatogram. Overlapped by the alanine spot, the dipicolinate area can be recognized by its strong absorption in the ultraviolet region and the slow development of a permanent blue color with ninhydrin. Acid hydrolysis of an eluate from the ultraviolet-absorbing area releases five other amino acids. These can be separated chromatographically from the dipicolinate which now no longer migrates as a band but moves as a discrete spot. Calcium appears to be essential for the maintenance of this "complex" between dipicolinate and amino acids. The possible significance of such a combination in the spore is discussed.

Introduction

Within the vegetative cells of many bacteria there are amino acids not in combination as proteins and peptides. These free amino acids can be completely extracted by boiling a suspension of cells in water for 15 minutes (3). A survey of the amino acid composition of several aerobic bacilli revealed that the resting spores of these species likewise contained free amino acids, but, unlike the pool of the vegetative forms, they could not be extracted by mere boiling. Quantitative extraction could be effected by prior disruption of the spores. However, subsequent attempts to separate the free amino acids of this spore extract by a paper chromatographic technique (9) were impeded by the presence in the extract of dipicolinic acid (pyridine-2,6-dicarboxylic acid), a compound unique to bacterial spores (7). This paper describes a relationship found between dipicolinic acid, calcium, and the free amino acids in extracts of spores of *Bacillus megaterium*.

Materials and Methods

Spores of *B. megaterium* were produced following vegetative growth of the organism on the surface of the agar medium (supplemented with 0.5% casamino acids) of Howie and Cruickshank (4). The spores were harvested following lysis of the sporangia and washed free of all vegetative debris as described previously (1).

Spores were disrupted by vibration of a suspension (2 to 4 mg dry weight of cells per ml) in the Mickle (5) tissue disintegrator at 2° to 4° C. The free

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amino acid pool was then extracted by heating the water suspension of disrupted spores at 100° C for 15 minutes. Centrifugation of this suspension at 10,000×*g* for 5 minutes yielded a supernatant (hot water extract) which contained the free amino acid pool.

Amino acids were resolved by ascending chromatography of a portion of the hot water extract on Whatman No. 1 filter paper with the two-dimensional solvent system of Redfield (9) (methanol 20, water 5, pyridine 1; *tert.*-butanol 10, methyl ethyl ketone 10, water 5, diethylamine 1). They were detected by spraying the dried chromatograms with ninhydrin (0.5% in 70% ethanol) followed by heating at 65° C until the characteristic blue color developed. Identification of the various amino acid spots was made by comparison of the position of the spots with those on a chromatogram of a standard solution of 18 amino acids resolved and developed under identical conditions.

Dipicolinate was identified by its characteristic absorption spectrum in the ultraviolet region using a Beckman model DU spectrophotometer (7). Total absorption at 270 mμ was used to estimate the amount of the compound in solution. Chromatograms were scanned in ultraviolet light using a Mineralite lamp (model SL 2537) to detect absorbing areas.

Results

Some Components of the Hot Water Extract

Six ninhydrin-positive spots were observed on the chromatograms of the hot water extract of the disrupted spores. They corresponded in position to arginine, glutamine, serine, alanine, aspartic acid, and glutamic acid. A few days after spraying, however, a further area which trailed through the chromatograms as a two-limbed band became slowly ninhydrin positive. Contrary to the color of the amino acid – ninhydrin complex, that of this complex did not fade. The distinctive blue color is still present after 2 years.

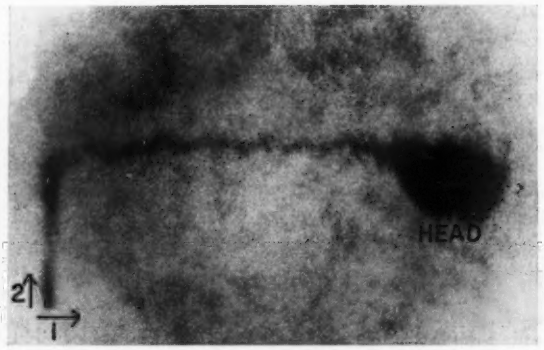

FIG. 1. Chromatogram of a hot water extract of disrupted spores of *B. megaterium* photographed in ultraviolet light. The absorbing region contained dipicolinic acid. Resolved by the two-dimensional solvent system of Redfield (9).

Chromatograms which were viewed in ultraviolet light prior to spraying with ninhydrin contained an area of strong absorbance which corresponded in position to the two-limbed band seen after spraying (Fig. 1). An absorption spectrum of an aqueous eluate of this area indicated absorption maxima at 263, 270, and 277.5 mμ, suggesting the presence of the calcium salt of dipicolinic acid (7).

That the dipicolinate was put into solution so readily was somewhat surprising, since Perry and Foster (6) had recently reported that it was necessary to boil intact spores in either 3 *N* sulphuric acid or 3 *N* sodium hydroxide (2) to ensure quantitative release of the compound. Some further experiments indicated that neither boiling nor acid or alkaline conditions were necessary for the complete release of the compound, provided that first the spores were disrupted (Table I). Following both cold and hot water extraction of disrupted spores (Table I) no dipicolinate could be detected in the spore residues. Even acid hydrolysis (6 *N* HCl at 100° to 110° C for 20 hours) of these residues failed to liberate detectable dipicolinate.

TABLE I

The effect of various extraction procedures on the quantity of dipicolinic acid released from resting spores of *Bacillus megaterium*

Treatment	% dipicolinate extracted*
1. Cold water extraction of whole spores	0
2. Hot water extraction (100° C for 15 min) of whole spores	85
3. Cold water extraction (2°–4° C) of disrupted spores	100
4. Hot water extraction (100° C for 15 min) of disrupted spores	100

*The absorption at 270 mμ of a suitable dilution of the hot water extract of disrupted spores was taken as 100%. Absorption at the same wave length of the other extracts derived from the same number of spores was expressed as a percentage of this value.

Further Observations on the Area Containing Dipicolinate

Since overloading of the chromatograms could lead to the tailing of dipicolinate, a series of chromatograms was set up with graded amounts (5 μl to 100 μl per paper) of a hot water extract. Tailing of the area absorbing in the ultraviolet still persisted even in the lower concentrations and although the limb to the origin became quite indistinct in ultraviolet light it still could be detected by the slow ninhydrin reaction.

Standard solutions of dipicolinic acid[3] subjected to the same procedures likewise became slowly ninhydrin positive, but showed no tailing effects on the chromatograms even at very high concentrations.

Only one amino acid (alanine) partly overlapped in the "head" region of the band containing dipicolinate. Thus a relative separation of the dipicolinate from the free amino acids could be achieved by marking, cutting, and eluting the ultraviolet-absorbing area. Rechromatography of this eluate (concentrated by lyophilization) produced only one discrete ultraviolet-absorbing spot with an R_f corresponding to the "head" region and the one ninhydrin-positive spot (alanine which had contaminated the eluate).

[3]The standard dipicolinic acid was kindly synthesized by Dr. G. D. Thorn of the Science Service Laboratory, London, Canada.

Hydrolysis.—A portion of the eluate was lyophilized and hydrolyzed in 6 *N* HCl for 20 hours at 100° to 110° C. Hydrochloric acid was removed by repeated drying *in vacuo* over calcium chloride and potassium hydroxide flakes. Chromatograms of the hydrolyzate in aqueous solution not only possessed a single ultraviolet-absorbing spot ("head" region) but also six ninhydrin-positive spots. These spots have been tentatively identified as α-ϵ-diaminopimelic acid, glutamic acid, tyrosine, valine, and isoleucine. Furthermore, eluates derived from various regions of the original two-limbed band each yielded the same amino acids upon hydrolysis and only one ultraviolet-absorbing spot in the "head" region.

These preliminary results strongly suggested that the dipicolinate was found in the spore in combination with amino acids or peptides. Some further investigations into the nature of this complex were carried out.

Dialysis Studies

A cold water extract (2°–4° C) of disrupted spores was dialyzed in the cold for 48 hours. Approximately one half of the total solids, including the free amino acids, escaped into the diffusate. Chromatograms of the diffusate (concentrated by lyophilization) contained a similar amino acid and dipicolinate pattern as the whole extract. As before, amino acids were liberated from the ultraviolet-absorbing areas following elution, hydrolysis, and rechromatography.

Spectral Analysis

Since no qualitative difference could be found in the amino acid composition of the various areas of the dipicolinate-containing band, the major segments were analyzed for any difference in their absorption spectra in the ultraviolet. Free dipicolinic acid and its calcium salt, subjected to the same procedures as the biological extract, were used as reference standards. No differences were noted between the various spectra; even the free acid upon elution from the chromatography paper was in the calcium salt form. Further studies indicated that Whatman No. 1 paper was indeed rich in calcium which could be removed only by rigorous washing of the paper. However, the spectrum of the free acid was not altered to the calcium form following elution from Schleicher and Schuell (S. & S.) No. 589 chromatography paper. Therefore this paper was considered relatively free of calcium.

Although substitution of S. & S. No. 589 paper for Whatman No. 1 did not alter the running characteristics of the ultraviolet-absorbing band, only the origin upon elution gave an absorption definitely characteristic of the calcium salt form. The other segments had no maximum at 277.5 mμ; rather, the spectra resembled that of the sodium or potassium salts.

Repeated Chromatography on Calcium-Free Paper

A portion of a hot water extract was applied to S. & S. No. 589 paper and the free amino acids and dipicolinate resolved as before. The ultraviolet-absorbing areas were cut and eluted. One half of the eluate was prepared for rechromatography and the other half for hydrolysis prior to rechromatography.

As before, a single discrete ultraviolet-absorbing area ("head" region) was found upon rechromatography of the eluates (both unhydrolyzed and hydrolyzed). However, unlike rechromatography on calcium-containing paper, a variable number and quantity of amino acids were released even without hydrolysis. Nevertheless consistently greater amounts were released following hydrolysis.

Separation of Standard Amino Acids from Standard Dipicolinic Acid

Standard solutions of dipicolinic acid and of a mixture of 18 amino acids were added as a single spot to both S. & S. No. 589 paper and to the same paper but which had been dipped previously in calcium acetate (0.1%) and dried. One discrete ultraviolet-absorbing spot ("head" region) was detected on each chromatogram following resolution. No tailing occurred. Furthermore all 18 amino acids could be identified on each paper after spraying with ninhydrin, and, as before, alanine overlapped the dipicolinate spot. These ultraviolet-absorbing spots were cut and eluted from unsprayed chromatograms and the eluate hydrolyzed before rerunning on untreated S. & S. No. 589 paper. The hydrolyzed eluate from the calcium-dipped paper released a full complement of the 18 amino acids when rechromatographed. On the other hand, the eluate from the non-calcium-dipped paper contained no detectable amino acids. No quantitative studies of this dipicolinate – calcium – amino acid complex have yet been made.

Discussion and Conclusions

It has been suggested by several authors that dipicolinic acid exists in the resting spore as a complex, in a bound organic or inorganic form (8, 6, 2). The only evidence that this might be so was the report of Powell and Strange (8) that dipicolinic acid is found in germination exudates in a chelate complex with calcium, and the inference of Perry and Foster (6) that the bulk of dipicolinic acid exists in a bound form since extraction of whole resting spores in boiling acid or alkali was essential to release the maximal amount.

The studies reported here provide some further evidence that dipicolonic acid may exist in resting spores in a combined form. The tailing effects observed with the dipicolinate from disrupted spore extracts and the lack of tailing with the synthetic solution under the same chromatographic conditions suggest that there is a difference between the two, perhaps in a degree of polymerization. However, such polymerization would need to be of small molecular dimensions since the dipicolinate is freely diffusible yet maintains the same chromatographic behavior following as before dialysis. Further, a complex dependent upon the presence of calcium appears to be formed between dipicolinate and amino acids. The amino acids in the complex are released by the removal of calcium and by acid hydrolysis. At the same time the chromatographic behavior of the spore dipicolinate changes to that of the synthetic compound. While in "combination" with the dipicolinate, the α-amino groups of the amino acids are masked or at least unable to combine with the ninhydrin to give the characteristic color.

The potential of dipicolinic acid and amino acids to form a complex in the presence of calcium (on filter paper) may not be of biological significance. Such a combination does suggest, however, a means by which dipicolinate could provide protective stabilization for essential proteins and/or nucleic acids.

Acknowledgments

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IMMUNE RESPONSES OF SOME INSECTS TO SOME BACTERIAL ANTIGENS[1]

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Abstract

Pseudomonas aeruginosa (Schroeter) Migula antigen remained in the blood of larvae of the wax moth, *Galleria mellonella* (L.), during the resistant period of the insect. Bacterial antigen present in the immune blood produces agglutinating titers in rabbits about 10 times as great as those produced by an approximately equal volume of standard *P. aeruginosa* vaccine. Attempts to demonstrate the mechanism that enhances the antigen showed that the active portion was contained in the serum, that the action occurred within several hours in vivo and only reached the same level after 3 days in vitro mixture, and that the action was probably not caused by lysis of the bacterial cells and the consequent liberation of more antigen in the blood. Electrophoretic studies on the blood mixture indicated that the altered or enhanced antigen may be bound to a blood fraction, the exact nature of which was not determined. The larvae were actively or passively immunized against lethal doses of *P. aeruginosa* within 20 to 24 hours. Concentration of vaccine had little effect upon the degree of immunity conferred upon the larvae. The immunity lasted about three days and was more specific than nonspecific. The larvae were not actively protected against *P. aeruginosa* by introduction of albuminous foreign material into the body cavity. True antibodies were not detectable in the immune blood though the bactericidal action of immune blood was at least twice as great as that of normal blood. Preliminary investigations on immune responses of other lepidopterous insects to *P. aeruginosa* antigen and of the wax moth to antigens of some other Gram-negative bacteria indicated similar results.

Introduction

It is of considerable interest in the study of vertebrate immunity to bacteria that after some bacterial antigens have been injected into wax moth larvae they show enhanced antigenicity for rabbits. This finding resulted from an investigation of the immune response in insects. Most of the work was done with a standardized antigen of *Pseudomonas aeruginosa* (Schroeter) Migula on adequate numbers of *Galleria mellonella* (L.), the test insect, and rabbits, the experimental mammal.

Research in insect immunity has been sporadic. Many of the results are conflicting and are impossible to duplicate, and the mechanisms that render insects immune have not been clearly understood. Early investigators rarely made any attempt to determine accurately dosage of antigen or of challenging microorganisms, and, moreover, worked only with small numbers of insects. Consequently it has been difficult to compare the immune responses of insects with those of mammals. Steinhaus (45) suggested that a study of insect immunity might have practical applications in vertebrate immunity, insect pathology, and medical entomology, but, because of lack of information on insect immunity, such contributions have not been made.

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Earlier investigators supported either the cellular or the humoral theories of insect immunity, and some attempted to reconcile the two theories. Metalnikov (30) concluded that bacteria fall into three groups insofar as insect immunity is concerned: group 1 contains bacteria to which complete immunity is shown, and includes most of the human pathogens; group 2 contains bacteria to which incomplete immunity is shown, e.g., *Staphylococcus* species; group 3 contains bacteria to which no natural immunity is shown, e.g., *Pseudomonas* species. He attributed the chief means of defense to phagocytosis, and later stated (34) that active immunization strengthens cellular activity. Paillot (38) suggested the hypothesis that antibodies are produced by phagocytic cells after adsorption of antigen, but later (39) concluded that natural immunity is probably of a dual nature and depends on both humoral and cellular factors. It has long been known that recovery from many diseases of vertebrates would be impossible without the co-operation of antibodies and phagocytes. Consequently, it is not illogical to deduce that a dual system functions in insects.

Several investigators (8, 14, 20, 32, 33, 37, 51), using various methods, demonstrated that insects could acquire active immunity to various bacteria. Wax moth larvae were immunized against *Escherichia coli* (Migula) Castellani and Chalmers and *Proteus vulgaris* Hauser more rapidly with small than with large doses of vaccine (35). Insects probably differ from mammals in this respect, though there are reports in the literature of mice that did not form detectable antibodies after injection of excessive doses of antigen (12). As with vaccines administered to mammals, oral administration of a vaccine did not protect insects (15).

Insects, like mammals, may acquire immunity passively: Zernoff (54) showed that injection of the blood of larvae immunized against Danysz bacillus, *Salmonella enteritidis* (Gaertner) Castellani and Chalmers provoked passive immunity in nonimmunized wax moth larvae, but that this immunity was not specific. He later demonstrated (55) that the immunity property appeared in leucocytes as well as in the plasma of immunized larvae. Findings contradictory to this are reported in the present paper.

Only three types of antibodies comparable to mammalian antibodies have been reported in insects: agglutinins (14), lysins (32, 37, 57, 58), and an antitoxin (9). Huff (22), in a review of immunity in invertebrates, questioned the use of terms used in mammalian immunology to describe antibodies in insects.

The purpose of this investigation was fivefold, namely, under more or less standardized conditions: (*a*) to determine whether larvae of the wax moth could be immunized against *P. aeruginosa* and whether antibodies similar to those produced by mammals were present in immune insect blood; (*b*) to study the fate of an antigen within the immune insect; (*c*) to study the nature of acquired immunity in the wax moth; (*d*) to determine whether larvae of the wax moth varied greatly in their responses to several bacterial antigens; and (*e*) to determine whether the immune responses of the wax moth varied greatly from those of several other insect species.

Materials and General Methods

Culture of the Wax Moth

Larvae of the wax moth, *G. mellonella*, were reared in the laboratory by the method of Peterson (40) on a medium composed of 908 g of Pablum (Mead Johnson of Canada Ltd., Belleville, Ontario), 260 ml of honey, 260 ml of glycerine, 130 ml of water, and 20 g of beeswax. At no time during this investigation was any disease noted in the rearing stock. Normal insects showed a low bacterial count in the gut. Mature larvae were selected as test insects by eye, all being within 3 to 5 days of forming cocoons. Experimental insects were maintained at 30° C in groups of 50 in ventilated glass jars, the mean weight for each group having been obtained.

Bacteriological Preparations

Vaccine for immunization tests was prepared as follows: Roux bottles were inoculated with *P. aeruginosa* and incubated at 37° C for 18 hours. The growth was emulsified in 25 ml of physiological saline, centrifuged, resuspended in fresh physiological saline, and diluted to a concentration of about 10^9 bacteria per milliliter by adjustment to an optical density of 0.280 to 0.320 on the Klett–Summerson photoelectric colorimeter. (McFarland Nephelometer tube No. 4 gives a reading of 0.304.) These suspensions were heat-killed at 56° C for 30 minutes. Each vaccine was tested for sterility before tests were made on experimental insects or mammals.

The antigen for use in agglutination tests was prepared as follows: growth of *P. aeruginosa* from a Roux bottle was suspended in 25 ml of 0.3% formolized saline, centrifuged, resuspended to the original volume of formolized saline, recentrifuged, and suspended in formolized saline to a concentration of about 10^9 cells per milliliter by adjustment on the colorimeter. The resultant suspension was incubated at 37° C for 18 hours to ensure the death of all bacteria.

Immunizing Techniques

To establish the pathogenicity of *P. aeruginosa* for the larvae, known doses of bacteria were injected into the body cavity of each larva and the median lethal dose calculated by the method of Reed and Muench (41). The LD_{50} for five groups of insects ranged from 6 to 13 bacteria. Ten times the LD_{50} usually killed 100% of the insects. There was no appreciable difference in pathogenicity for wax moth between the strains of *P. aeruginosa* so most insect vaccinations were made with P11-1 culture, a strain highly pathogenic for grasshoppers.

The antigenicity for rabbits of strains of *P. aeruginosa* isolated from grasshoppers (5) and of the American Type Culture Collection strain 10145 was investigated before attempts were made to immunize larvae against *P. aeruginosa*. Mature male rabbits were each inoculated in the marginal vein of the ear with 0.2, 0.4, 0.6, 0.8, and 1.0 ml of *P. aeruginosa* vaccine at intervals of 5 days. A rabbit with an average weight of 4 lb received in all about 3×10^9 dead bacteria, or about 1.6×10^6 dead bacteria per gram of weight. Five

days after the last injection each rabbit was bled. The formolized antigen was mixed with doubling dilutions of the antisera as in the conventional tube method (7). Test mixtures were incubated at 37° C for 2 to 4 hours, and the final readings were made after the tubes had remained in a cold room at 4° C for about 16 hours.

To check on the immunizing properties of the vaccine for wax moth larvae, groups of larvae of *G. mellonella* were challenged at regular intervals after vaccination with doses of *P. aeruginosa* at least 10 to 100 times as great as the LD_{50}. The larvae were injected with 0.01 ml of vaccine in the area of the third to fifth abdominal legs by a 30-gauge needle mounted on a Dutky-type microinjector. Repeated injections were undesirable because of the size of the insect. The mean weight of a larva was about 0.168 g, so that each larva received about 6×10^7 dead bacteria per gram of weight. Therefore, per unit of weight, each larva received about 50 times the dosage given each rabbit. A group of control insects was always injected with a dose equivalent to that used on the challenged insects.

Serological Tests on Blood of Immune Wax Moth Larvae

Agglutination tests were made by the conventional tube method (7) and in small tubes with all materials measured in lambdas rather than in milliliters. Microscopic slide agglutination tests were carried out with *P. aeruginosa* antigen tested against both normal and vaccinated wax moth blood.

Ring tests and simple mixture tests (7) and Oudin's agar column test (36) were performed on immune blood to test for the presence of precipitins. A modification of Roberts and Jones' method (43) for detection of minute traces of antibody was used on immune wax moth blood as follows: dead cells from a saline suspension of *P. aeruginosa* were centrifuged and the supernatant discarded; the cells were mixed in a 1:5 ratio with normal wax moth blood and incubated at 37° C for 10 to 16 hours; and the mixture was recentrifuged and the cells washed twice in physiological saline and brought to the original volume. These coated cells were used as antigen and mixed with 1:1 diluted vaccinated wax moth serum.

Fate of an Antigen in Blood of Immune Wax Moth Larvae

Collection and Preparation of Immune Insect Blood for Rabbit Injections

Large wax moth larvae were vaccinated as described, and pooled blood was collected after 20 to 24 hours by cutting a proleg from each larva and collecting one small drop of blood. The blood removed from a larva was not a large part of the total volume, for the larvae that had blood samples removed for such experiments survived, completed development, and reproduced in the normal manner. The mean weight was calculated for each group of larvae used for blood collections. On the assumption that the blood comprised about 41% of the total weight (42), that the specific gravity was 1.055 (21), and that all the antigen injected remained in the blood, the blood volume per wax moth was estimated and also the volume of antigen present in a given volume of pooled wax moth blood. The volume of antigen (in

the form of vaccine) in 0.5 ml of vaccinated blood ranged from 0.052 to 0.065 ml or in terms of numbers of bacteria from 5.2×10^7 to 6.5×10^7.

In most of the experiments, rabbits were injected intravenously with 0.5 to 1.0 ml of the pooled vaccinated blood, i.e. with from 5.2×10^7 to 1.3×10^8 bacteria. Rabbits injected with 0.5 to 1.0 ml of the slightly viscous blood of nonimmunized larvae showed no reactions to the introduction of the foreign material. All experimental rabbits were bled on the fifth or sixth day after injection. Control rabbits were injected with a dose of *P. aeruginosa* vaccine comparable to, or greater than, that estimated to be present in the volume of immune wax moth blood injected into experimental rabbits. Control rabbits were usually bled on the fifth day, and at 4- to 5-day intervals until the titer reached its maximum.

Tests for Adjuvant Action

Samples of vaccine were mixed with normal wax moth blood in proportions comparable to those in in vivo mixtures of wax moth blood and were tested for antigenicity to rabbits both immediately and at various intervals after mixing. The titers produced by rabbits in response to these mixtures were compared with those produced in control rabbits in response to comparable amounts of *P. aeruginosa* vaccine only.

Lysis of Antigenic Material

A preliminary attempt to lyse *P. aeruginosa* cells was made as follows: the cells from 2 liters of 16-hour nutrient broth (Difco) cultures of *P. aeruginosa* were centrifuged to remove extraneous material, washed twice in physiological saline, and resuspended in 200 ml of physiological saline. The resulting mixture was extremely turbid. It was treated in a sonic oscillator (Raytheon 10 kc) for about five minutes. The turbid, slightly orange-red, milky supernatant was used in experiments and is referred to here as the *sonate*. The *sonate* was not sterile, though only the undiluted preparation was lethal to wax moth larvae after an injection of 0.01 ml per larva.

Two rabbits were injected with 0.5 ml of the concentrated *sonate* and 6 days later they were bled and the agglutination titers determined. Similarly, four rabbits were injected with 0.4 ml of pooled blood from each of four groups of wax moth larvae 24 hours after they were injected with 0.01 ml of the 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions, respectively, of the *sonate*.

In an attempt to introduce disintegrated dead *P. aeruginosa* cells only, a Mickle disintegrator was used to rupture the cells. Two parts of *P. aeruginosa* vaccine were mixed with one part of ballotini beads in the Mickle chambers. The machine was set for maximum amplitude, and the material was oscillated for 2 hours at room temperature. The suspension was filtered through a fritted glass filter of coarse porosity, to remove the glass particles, and the filtered suspension was examined microscopically. The suspension was composed mainly of cell fragments and no intact bacteria could be seen. The antibody response in rabbits after an injection of this "disintegrated" vaccine was compared with that in rabbits injected with standard *P. aeruginosa* vaccine.

Attempts to Isolate Enhanced Antigenic Material from Blood of Wax Moth Larvae

By electrophoresis.—To investigate the possibility of *P. aeruginosa* antigen being bound to a component of wax moth blood, an attempt was made to separate the blood components by zone electrophoresis in a starch medium. The method of Kunkel and Slater (26) was used, with modifications devised by Dr. J. Robinson (Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Canada). Because of certain changes in procedure and because of the discovery that one of the fractions contained the enhanced antigen, the method is reported herein in more than customary detail. Potato starch (Hatfield Industries, Hartland, New Brunswick) was washed six times in water to remove extraneous material, washed over suction with alcohol and acetone, and dried at 45 to 50° C until no odor of alcohol or acetone was detectable. Tris(hydroxymethyl)aminomethane buffer[3] was added to the dry starch in an approximate proportion of 310 g of starch to 290 ml of buffer; this mixture was of a consistency at which the starch was barely pourable. A perspex block of suitable thickness (depending on the size of the sample) was inserted 30 cm from the anode end of a perspex column 75×15×12 cm. Buffer-soaked pads of Whatman 3 MM filter paper, 15 cm in length and 12 cm deep, were inserted about 5 cm from each end of this column. The column was then filled with the starch mixture and leveled with a spatula to ensure a good seal when the top was in position. The block was removed and the area filled with the test mixture (from 0.8 to 1.0 ml of vaccinated wax moth blood mixed with dry starch to a consistency comparable to that in the column). The lid of the column, a flat piece of perspex $\frac{1}{8}$ in. thick and of the exact dimensions of the trough, was thinly coated with stopcock grease before it was eased into position. The sides of the lid and trough were lightly smeared at the join with anhydrous lanolin to prevent diffusion. G-clamps were used to hold the top tightly in place. Connection of starch with buffer was made by the buffer-soaked pads in the column, and two-ply facial tissue bridges connected the dishes containing the pads with those holding the electrodes. The experiment was carried out at 300 volts for 18 to 20 hours, after which the block was cut into 15-mm segments. Each segment was eluted in 2 ml of Tris buffer. Protein determinations were made on 0.1-ml samples of the eluted material by the method of Lowry *et al.* (29).

By protein precipitation.—To investigate the possibility of antigen adherence to a blood protein, proteins were removed from immunized wax moth blood by the method of Somogyi (44), and, after centrifugation, the clear, protein-free supernatant was injected into a rabbit to test it for antigenicity. The protein precipitate was resuspended in saline to its original concentration and a portion of this injected into a second rabbit. Duplicate experiments were made with each of the two preparations.

[3]4.5 g of tris(hydroxymethyl)aminomethane; 0.45 g of $MgSO_4.7H_2O$; 1 liter of H_2O; pH adjusted to 7.3 with concentrated HCl and solution diluted to 1500 ml.

Active and Passive Immunization of Wax Moth Larvae

All materials used in the investigation on protective immunity were prepared as previously described. The method of vaccination was as described. Modifications of technique that were necessary are included with the account of experimental results.

Immunization of Wax Moth Larvae and Search for Antibodies

Table I shows the agglutination titers in rabbits against seven strains of *P. aeruginosa.* Agglutination was light and was of the floccular H type. None of the experimental rabbits before immunization showed a titer as high as 1:20 against the test strains of *P. aeruginosa.* The six insect strains of *P. aeruginosa* appeared to be closely related antigenically, but the American Type Culture Collection strain 10145 was antigenically distinct from them. The titers of homologous antisera and antigens did not exceed 1:2560, but the results compared favorably with those of Fox and Lowbury (13), who obtained titers of 1:5120 usually but in some cases only of 1:2560.

TABLE I

Agglutination titers in rabbits against seven strains of *P. aeruginosa*

	Antigen						
Antiserum	P11-1	1-1A	46-4B	251-4-1*a*	251-4-3	344-1A	A.T.C.C.* strain 10145
P11-1	1:2560	1:2560	1:1280	1:1280	1:1280	1:1280	0
1-1A	1:2560	1:2560	1:1280	1:640	1:1280	1:1280	0
46-4B	1:1280	1:1280	1:2560	1:640	1:1280	1:640	0
251-4-1*a*	1:1280	1:1280	1:1280	1:1280	1:1280	1:1280	0
251-4-3	1:1280	1:1280	1:1280	1:1280	1:1280	1:1280	0
344-1-A	1:1280	1:1280	1:1280	1:1280	1:1280	1:1280	0
A.T.C.C.* strain 10145	0	0	0	0	0	0	1:1280

*All strains from grasshoppers except American Type Culture Collection strain 10145.

Table II shows that immunity developed rapidly in vaccinated larvae and reached a maximum in 12 to 24 hours, when only 15 to 20% mortalities were observed. The immunity decreased slightly in the 24- to 32-hour period and more markedly in the 32- to 48-hour period. Few larvae were immune 3 days after vaccination. As the level of immunity was largely constant from 12 to 24 hours after vaccination, most further studies on the immune responses of insects were made during this period.

As *P. aeruginosa* vaccine gave rise to agglutinins in rabbits (Table I), preliminary experiments on antibody detection were made for the presence of agglutinins in blood of the immune wax moth larvae. The conventional tube agglutination method (7) yielded consistently negative results, as did agglutinations carried out in small tubes. Slide agglutination tests showed heavy aggregates of cells and bacteria within 1 to 5 minutes in the mixtures with vaccinated blood whereas no aggregates were present in the normal

TABLE II

Percentage mortalities among groups of 40 larvae of *G. mellonella* after lethal doses of *P. aeruginosa* at various intervals after vaccination with 0.01 ml of *P. aeruginosa* vaccine

Time after vaccination, hours	Dose in bacteria per larva	Mortality, %*
2	120	90
4	87	80
6	105	60
8	133	40
12	252	20
16	210	20
20	203	15
24	210	15
32	231	30
48	280	75
72	196	80

*100% mortality occurred in equal numbers of control insects.

blood mixtures though in about 10 minutes some clumping occurred among the normal blood cells. Though aggregations in normal blood never approached the intensity of those in vaccinated blood, it is doubtful that this phenomenon can be considered a true agglutination reaction such as is known in mammals, for more probably some other factors exist in vaccinated blood that stimulate clumping of cells and bacteria. Similar tests were not carried out on immune serum because only limited quantities of blood were available. Glaser (14) asserted that true agglutinins were present in grasshopper blood.

Ring tests (7), simple mixture tests (7), and Oudin's agar column test (36) for precipitins gave negative results. As tests for agglutinins and precipitins in the wax moth proved negative, and as *P. aeruginosa* vaccine protected the wax moth larvae, it was possible that antibodies were present in amounts too small to be detectable by ordinary techniques. Therefore a modification of Roberts and Jones' method (43) for detection of minute traces of antibody was used on immune wax moth blood. Agglutination was noted when the coated cells, used as antigen, were mixed with diluted vaccinated wax moth serum, but no agglutination occurred when a similar test was made with normal wax moth serum. The implications of these results are not clearly understood but it is possible that the antibody in wax moth blood may be present in so small quantity that a sensitized, coated antigen is necessary for its detection.

Tests for complement in the blood of *G. mellonella* have not yet been attempted. No tests were made for antitoxin production in the blood of wax moth larvae as little is known of the ability of *P. aeruginosa* to produce an exotoxin.

Bacteriolysins were demonstrated in insect blood by several investigators (32, 37, 57, 58); most workers believe that these are the most common and active antibodies in insects. No bacteriolysins were demonstrated in the blood of wax moth larvae. When 5 ml of blood of normal or vaccinated wax moth larvae was added to 5 ml of an 18-hour culture of *P. aeruginosa*, no lysis or

clearing of the culture was evident but a reduction in numbers of cells was observed, about 20% with normal blood and 40% with vaccinated blood.

Briggs (4) referred to a heat-stable antibacterial principle that is active in the blood of lepidopterous insects a few hours after vaccination. The bactericidal effect of immune wax moth blood on *P. aeruginosa* in pour plates was at least double that of normal wax moth blood. It has not been determined definitely whether this is due to the same principle described by Briggs. Studies on the bactericidal effect of normal and immune wax moth blood will be reported in a later publication.

Fate of an Antigen in Blood of Immune Wax Moth Larvae

Preliminary investigations were made on the survival and disposition of the antigen within the insect as the same antigen that produced antibodies in rabbits provoked an immune state without demonstrable antibodies in wax moth larvae. The results indicated that within the insect blood the antigen was in some way enhanced in its antigenicity for rabbits. An intensive study of this phenomenon was therefore made.

It has been stated, by investigators of mammalian immunity, that detectable antibody generally does not appear in the blood until antigen has disappeared (48); the period between antigenic stimulation and initial antibody production in man may vary from 4 to 21 days. Apparently no intensive studies have been made on the fate of an antigen after its introduction into an insect though it is thought that insects do not readily produce antibodies in response to antigenic stimulation. If the immune response in an insect follows the same general pattern that occurs in mammals, one would expect that the antigen would be eliminated very rapidly from the blood, as immunity is developed within 24 hours. On the assumption that it would be difficult to measure the rate of elimination of antigen from the insect blood, a preliminary test was made to determine whether any antigen was present in the blood of vaccinated wax moth larvae 24 hours after immunization. The blood of a rabbit injected with 0.5 ml of whole blood from immunized wax moth larvae was checked for the presence of antibodies to the *P. aeruginosa* antigen. Remarkably enough, within 5 days the rabbit produced a moderately high agglutination titer in comparison with the amount of original antigen that was calculated to be present in the 0.5 ml of immune wax moth blood. Consequently, further tests were made to determine whether the preliminary finding was due to chance, and to investigate the mechanisms responsible for the action.

Antigenic Enhancement in Insect Blood

Further tests indicated that the antigenicity of *P. aeruginosa* for rabbits was generally enhanced in the blood of wax moth larvae 24 hours after vaccination (Table III). None of the untreated rabbits showed antibody response at the 1:20 dilution. One to two hours after vaccination of the wax moth, the antigen within the blood produced an antibody response in a rabbit

at least 4 times as great as a comparable dose of control vaccine; 24 hours after vaccination, 10 to 12 times as great. At 2 to 4 hours after vaccination the larvae were not protected against challenge doses of *P. aeruginosa* but the blood already showed increased antigenicity for rabbits. Antigenicity was still great at 24 to 48 hours, but at 72 hours, when immunity in the wax moth had almost disappeared, it was no greater than with control doses of *P. aeruginosa* vaccine. The results suggest a connection, not yet understood, between the time of increased antigenicity and that of maximum protection (Table II) of the insects against the bacteria. Moreover, the antigen must evidently remain in the wax moth larvae in a concentration at least equal to the original during the time at which maximum resistance is found in the larvae. If the immune pattern in insects is similar to that in mammals, it is not surprising that antibodies were not found when so great a concentration of vaccine was present in the blood during the time of maximum resistance of the insect.

TABLE III

Agglutination titers in experimental rabbits after injection with *P. aeruginosa* antigen in blood of immune wax moth larvae and in control rabbits after injection with standard antigen*

Experimental rabbits					Control rabbits		
Rabbit No.	Hours after vaccination when blood removed from insects	Treatment of immune insects and special treatment of insect blood before injection into rabbits	Estimated original volume of antigen, ml†	Titer	Rabbit No.	Volume of antigen, ml	Titer
1	1		0.065	160	31	0.2	80
2	2		0.065	160	32	0.2	80
3	6		0.065	320	33	0.07	40
4	24		0.060	160	34	0.07	20
5	24		0.065	320	35	0.7	320
6	24		0.065	320	36	0.8	640
7	24		0.104	640	37	1.0	640
8	24		0.100	640	38	1.0	640
9	45		0.057	160			
10	48		0.065	320			
11	72		0.057	80			
12	72		0.065	80			
13	72		0.065	40			
14	24	0.01 ml of dilute vaccine	0.060	320			
15	24	0.0067 ml of standard vaccine	0.060	320			
16	24	0.01 ml of concentrated vaccine	0.020	80	39	0.2‡	80
17	24	Serum only injected into rabbit	0.065	320			
18	24	Blood cells from above in normal saline to rabbit		20			
19	24	Serum only injected into rabbit	0.060	320			
20	24	Blood cells from above washed twice in normal saline before injection to rabbit		20			
21	24	Blood heated to 60° C for 30 minutes	0.065	40			
22	24	Blood stored at 5° C for 4 days before injection	0.098	320			
23	24	Blood of vaccinated insects that survived challenge dose of *P. aeruginosa*	0.013	160			
24	24	Blood of vaccinated insects that survived challenge dose of *P. aeruginosa*	0.065	1280			
25	24	Supernatant of standard vaccine per insect	0.065	160	40	0.5§	160
26	24	Standard vaccine treated 2 hours in Mickle disintegrator	0.052	320	41	0.2‖	80
27	24	Rabbits injected with series of doses of wax moth blood of increasing volumes	0.360	1280			

*Each wax moth larva was injected with 0.01 ml of standard vaccine except those used to inject rabbits 14–16. The pooled blood of immunized insects was not specially treated before injection into rabbits unless stated.
†In volumes of insect blood ranging from 0.1 to 1.0 ml — 1 ml antigen equivalent to 10^9 cells.
‡Concentrated vaccine.
§Supernatant of vaccine.
‖Vaccine treated for 2 hours in Mickle disintegrator.

It is noteworthy that in the rabbit all responses to antigen within wax moth blood were evident and at their optimum within 5 days. Moreover, a single injection of the blood mixture was just as efficient in producing antibodies as a comparable volume given in several small doses 4 to 5 days apart. In most cases it was necessary to wait at least 7 days to demonstrate antibody production in the control rabbits; for example, rabbit No. 37 was given a single injection of 1.0 ml of *P. aeruginosa* vaccine but the final titer was not reached until the 12th day, whereas with rabbits Nos. 4, 5, and 6 titers of 160 and 320 were reached in 5 days with one injection only, and with rabbits Nos. 7 and 8 a titer of 640 was reached. It is known that above the minimal threshold value, the titer varies with the dose administered but the increase in titer is smaller than the increase in dose required to produce it (52). From Table III it appears that the increase in titer to *P. aeruginosa* in rabbits may be proportional to the dose of antigen within immune wax moth blood. Rabbits only reached maximum titers of 1:2560 after five injections of increasing volume in a straight course of immunization with *P. aeruginosa* vaccine (Table I). The presence of wax moth blood in combination with the *P. aeruginosa* vaccine evidently reduced the number of injections required and increased the antibody response.

Tests to determine whether the antigen was enhanced only in the presence of the whole blood or whether the cells or serum alone could produce the same phenomenon showed that the serum gave the greater antibody response (Table III). That the 1:20 titer with cells in rabbit No. 18 was probably caused by slight adherence of antigenic material to the cells and not by contamination due to improper centrifugation was shown by the tests with rabbits No. 19 and No. 20, for which cells from the immune blood were washed twice to remove any traces of serum. This suggests that, contrary to earlier findings (6, 19), the cells do not ingest the bacteria in the vaccine, or, if they do, that the active antigenic material must be released in the serum. Microscopic examination of wax moth blood 24 hours after vaccination showed many more *P. aeruginosa* cells free in the serum than ingested by or adhering to blood cells.

The concentration of vaccine had little effect on the final antibody titer produced by rabbits against the antigen–blood mixture. Though rabbit No. 16, which was injected with concentrated vaccine, did not receive a dose comparable in size to that of rabbit No. 14 because of an insufficient supply of wax moth blood, the increase in response over that of the control was still in the 10-fold range. It is still not understood how the wax moth blood increases the efficiency of the smaller number of cells in the antigenic material.

Though the antigen – wax moth blood mixture lost its increased antigenicity after 72 hours within the insect, storage of immunized blood for several days had no effect upon the antigenicity, nor had freezing of the blood mixture for a month or longer. Heating the immune blood to 60° C for 30 minutes appeared to decrease the antigenicity (Table III). This suggests that

denaturation of a protein or proteins may be in part responsible for loss of antigenicity, and thus may indicate that the antigen is connected in some way with one of the proteins in the wax moth blood.

The antibody response to *P. aeruginosa* in wax moth blood developed rapidly in the rabbit but appeared to persist longer than immunity to standard vaccine at the same titer. For example, the blood of control rabbits showing original titers of 1:320 to *P. aeruginosa* vaccine showed titers of 1:20 3 months later, whereas the blood of rabbits injected with the vaccine–blood mixture and showing original titers of 1:320 showed titers of 1:160 3 months after the original injection and 1:80 4 months after. Therefore the use of a vaccine–blood mixture may possibly prolong the antibody response, in addition to increasing it and decreasing the number of injections needed.

"Booster" inoculations against many bacteria are said to give as good results in man as a customary series of inoculations for an original immunizing schedule (7). Immunized rabbits whose antibody titers had dropped from 1:320 to 1:20 were given a "booster" injection of 0.1 ml of immunized blood; this amount of blood probably contained about 0.013 ml of *P. aeruginosa* vaccine or 1.3×10^7 bacteria. The titer once again reached the 1:320 level 5 days after the "booster" injection. A "booster" injection of 0.02 ml (2×10^7 bacteria) of standard vaccine into two control rabbits, whose antibody titers had dropped to the 1:20 level, brought the antibody level to 1:40 and to 1:80. Again, increased antigenicity of the vaccine within wax moth blood was suggested, as the "booster" of wax moth blood appeared more efficient than the control.

Table III also shows that the blood of vaccinated larvae which had survived a dose of *P. aeruginosa* large enough to cause 100% mortality in nonvaccinated controls was even more greatly increased in antigenic power than the non-challenged vaccinated blood, as 0.065 ml was responsible for a titer of 1:1280 in rabbit No. 24. The insect blood was examined microscopically before its injection into the rabbit, but no living bacteria were found free in the serum, and very few bacteria were observed phagocytosed by the blood cells. To exclude the possibility of multiplication of living *Pseudomonas* in the larval blood increasing the dose of bacteria given, cultures were made from the blood of living larvae; 1 ml of this blood was cultured in 5.0 ml of Bacto nutrient broth (Difco) and incubated at 37° C for 72 hours but *P. aeruginosa* was not detected. The further increase in antigenicity is apparently due not to living cells but to some other mechanism that is evidently stimulated by the presence of living bacteria.

Mechanism That Enhances the Antigen

Possibility of Action of an Adjuvant

Use of additional substances for intensification of the antibody response has been recognized and studied for some time (3). Such adjuvants may be antigenic or nonantigenic. Though the mechanism of action of adjuvants is not entirely understood, Boyd (3) states that when they bring the antigen into particulate form they may influence its being taken up by the phagocytes

TABLE IV

Agglutination titers in rabbits after administration of in vitro mixtures of *P. aeruginosa* vaccine and normal wax moth blood and in control rabbits after comparable doses of *P. aeruginosa* vaccine only

In vitro mixture					*P. aeruginosa* vaccine		
Rabbit No.	Time of injection to rabbit	Vol. blood, ml	Vol. vaccine, ml*	Titer	Rabbit No.	Vol. vaccine, ml	Titer
51	2 hr after mixing	0.5	0.060	40	71	0.07	20
52	18 hr after mixing	0.5	0.065	80	72	0.07	20
53	24 hr after mixing	0.5	0.065	80	73	0.07	20
54	72 hr after mixing	0.4	0.060	160	74	0.06	20
55	Rabbit first injected with wax moth blood and 2 hr later with vaccine	0.5	0.065	80	75	0.07	20

*1 ml equivalent to 10^9 cells.

and that such precipitated antigen may persist in the tissues longer than an unaccompanied antigen. As wax moth blood is a viscous and presumably antigenic material, an investigation was conducted on whether the enhanced antigenicity of the mixture was caused by its action as an adjuvant.

Table IV indicates that the wax moth blood does not act as a true adjuvant as at 2 hours, and even 24 hours after mixing, the antibody response to the mixture was little better than that to control vaccine. However, it is noteworthy that an increase in antibody response occurred with time in the in vitro mixture whereas the response is much more rapid within the insect body. A reaction possibly occurs within the insect which takes only a few hours to complete whereas in an in vitro mixture the action takes place slowly and perhaps is not fully completed in 3 days. Therefore, some mechanism other than an adjuvant action of the blood of wax moth larvae is evidently largely responsible for the increase in antigenicity of *P. aeruginosa* vaccine.

Effects of Lysing Antigenic Material

The increased antigenicity of *P. aeruginosa* vaccine within wax moth blood suggests that lysis of the bacterial cells may occur, thereby making a greater surface of antigenic material available to promote antibody production. Though attempts to demonstrate a bacteriolysin in the blood of immune wax moth larvae by the usual methods were negative, the possibility of the existence of a lytic factor not demonstrable by ordinary techniques still remained. Several attempts were made to lyse *P. aeruginosa* in the sonic oscillator and to compare the antibody response to the lysed material with that to control vaccine.

Two rabbits injected with 0.5 ml of the concentrated *sonate* each gave an antibody titer of 1:1280 in 6 days. This titer is higher than that obtained with an equivalent amount of standard *P. aeruginosa* vaccine (Table III) but, as living cells were present, the increase in titer should not be directly attributed to the effect of lysis on the bacteria.

Of the four rabbits injected with blood from wax moth larvae that had received dilutions of *sonate*, the one receiving the 10^{-1} dilution was dead from *P. aeruginosa* infection 3 days after injection. The three remaining rabbits were bled on the sixth day and their blood gave the following titers: 1:640 for the rabbits with 10^{-2} and 10^{-3} dilutions and 1:320 for the one with a 10^{-4} dilution. Though the 10^{-1} dilution that was passed through the insects without causing their death was responsible for the death of the rabbit, a large dose of undiluted *sonate* had no effect on a fifth rabbit. It is concluded that the sonic treatment of *P. aeruginosa* is not adequate for testing effects of lysis on increased antigenicity, as it is impossible to lyse all the cells by this method.

A Mickle disintegrator was used in an attempt to lyse the vaccine in case increased antigenicity was due to lysis of the dead bacterial cells. As shown in Table III, lysis of the vaccine had no appreciable effect on the antibody titer. The blood of wax moth larvae injected with this "disintegrated" vaccine and given to rabbit No. 26 showed little change in antigenicity. This suggests that ordinary lysis of the *P. aeruginosa* cells within the wax moth blood is not responsible for an increase in antigenicity, and that the increase may be caused by an altered antigen, perhaps even by chemical combination.

Attempts to Isolate Enhanced Antigenic Material from Blood of Immune Wax Moth Larvae

By Electrophoresis

The possibility of a combination of *P. aeruginosa* antigen or a component with an insect blood fraction was discussed in the preceding section. Krieg (25), using paper electrophoresis, reported no binding of the *Staphylococcus aureus* (=*Micrococcus pyogenes* var. *aureus* (Rosenbach) Zopf) antigen on the blood fractions of several insect species. Preliminary tests by Barlow (1), who used paper electrophoresis, suggested an extension of one of the blood fractions in vaccinated individuals, although results were not always consistent. An attempt was made to separate the blood components by zone electrophoresis on a starch medium.

In the first experiment with vaccinated wax moth blood a series of eight rabbits were injected with 1 ml of the eluted material from the first eight sections, moving from the origin to the cathode. Rabbit No. 1, injected with material one section removed from the origin, showed a high antibody response in comparison with the calculated amount of antigen in immune wax moth blood (Table V). The response was greater than when a comparable amount of antigen in immune wax moth blood was injected into a rabbit. The active fraction was usually found in the first two sections next to the origin; in these areas the protein determinations were not as high as in sections nearer the cathode.

The speed of movement of insect blood fractions more closely resembles that of the globulin rather than that of albumin fractions of vertebrate blood. The fact that the antigenic material was always connected with a section near the origin suggests, however, that it may not necessarily be bound to a

TABLE V

Antibody response in rabbits to eluted material from electrophoretic fractionation of vaccinated wax moth blood

Test No.	Vol. eluate injected, ml	Section from which eluate derived*	Calculated vol. antigen, ml†	Antibody titer
1	0.5	1	0.029	320
2	0.5	1	0.020	320
3	1.0	2	0.020	320
4	0.75	1	0.015	160–320

*Number of sections from origin toward cathode.
†1 ml antigen (vaccine) equivalent to 10^9 cells.

protein fraction, as the greater concentration of protein is situated at a greater distance from the origin. An orange color was noted on several occasions on the fraction containing the active component. The significance of the color was not determined.

Protein Precipitation

Preliminary attempts were also made to determine the nature of the material to which the antigen adhered. Duplicate experiments on injecting rabbits with the protein-free portion and the protein precipitate of immune wax moth blood showed an antibody response to the precipitate but not to the supernatant. This evidence, though not conclusive, suggests that the antigen adheres to or is bound to a blood protein within the immune insect blood.

Protective Immunity in the Wax Moth

A detailed investigation on actively and passively acquired immunity and on the properties of the immune blood is reported below in considerable detail, as few investigations of this nature have been reported since the 1930's, when most workers did not use modern or reproducible techniques and, moreover, worked with insufficient numbers of insects.

Active Immunization

A series of tests were made on groups of wax moth larvae to compare the mean mortality in groups of larvae 24 hours after vaccination with that in groups of nonvaccinated individuals. Table VI shows that though vaccination with a homologous vaccine did not give 100% protection, the percentage mortality in vaccinated insects was considerably less than that in the control insects. Early investigators often tested only 5 or 10 specimens, and did not attempt to calculate the percentage mortality. It appears that some investigators (51, 60) did not obtain survival rates as good as reported herein.

Many of the early investigators refer to immunity acquired in 24 hours but of short duration (34, 35). However, Zernoff (59) referred to specific immunity in *Carausius morosus* Brauer that lasted several weeks, and stated that repeated vaccinations increased the resistance. Two doses of vaccine

TABLE VI

Percentage mortalities from lethal doses of *P. aeruginosa* in normal wax moth larvae and in wax moth larvae vaccinated with 0.01 ml of vaccine 20 to 24 hours before injection of culture

	No. of larvae in each group	Dose in bacteria per larva	Mortality, %	
			Vaccinated	Control
	40	1050	20	100
	40	1300	35	90
	40	1150	44	100
	40	925	26	100
	40	700	40	100
	40	980	26	100
	95	700	19	100
	40	2100	23	95
	35	350	37	100
	30	1190	20	100
	95	210	32	95
	80	945	34	100
	100	154	18	100
	100	280	37	100
	100	910	38	100
Mean	61	863	30	99

did not appear to increase the resistance of wax moth larvae to *P. aeruginosa*; moreover, the concentration of vaccine probably had less effect on the degree of immunization in insects than in mammals. Forty-three per cent mortality was observed in a group of wax moth larvae vaccinated with 0.0067 ml of vaccine and 100% in the control group after a challenge dose of 180 bacteria. In a group of larvae that received 0.01 ml of vaccine containing twice the normal number of cells, 50% mortality occurred after a challenge dose of 104 *P. aeruginosa*, whereas 100% mortality occurred in the control group. Zernoff (60) found that large doses of Danysz bacillus vaccine sometimes sensitized wax moth larvae and that they became less resistant to the bacteria than were normal insects. He also stated that dilution of the vaccine plays a secondary role, which is in accord with the present results.

The mortalities from *P. aeruginosa* show that there may be considerable variation from population to population. For example, 44% mortality occurred in vaccinated individuals challenged with a dose of 1150 bacteria and 100% mortality in controls. With an almost twofold dose of 2100 bacteria only 23% mortality occurred in the vaccinated individuals and 95% in the controls. It must not be assumed, however, that any of the deaths were caused by mechanical injuries, as wax moth larvae withstand at least three or four injections of nontoxic materials such as *P. aeruginosa* vaccine, Bacto nutrient broth (Difco), or physiological saline. These individuals develop normally and produce apparently normal adults.

Passive Immunization

Zernoff (55, 56) showed that larvae of *G. mellonella* could be passively immunized against Danysz bacillus by injection of whole blood, leucocytes,

or serum but that exposure of the vaccinated blood to high temperatures destroyed its immunizing properties. As Zernoff (53, 54, 56) was unable to confer the same degree of immunity to the wax moth by injection of the blood of normal larvae or by the blood of larvae injected with nonspecific substances such as broth, he concluded that passive immunity was relatively specific and was probably caused by a simple defense reaction provided by a stimulation such as the injection of immune blood.

To test the possibility of passively immunizing larvae of *G. mellonella* against *P. aeruginosa* seven groups of larvae were each vaccinated with 0.01 ml of P11-1 vaccine, and at seven different time intervals, pooled samples of blood were obtained. Normal larvae in a second series were each injected with 0.01 ml of the pooled blood and 20 to 24 hours later were given a challenge dose of P11-1. Individuals of an eighth and a ninth group were injected, before a challenge dose, with 0.01 ml of a saline suspension of cells from vaccinated larvae and with 0.01 ml of serum of blood of vaccinated larvae respectively. An attempt was made to immunize larvae of a 10th group passively by injection of vaccinated blood heated to 60° C for 30 minutes after its removal from the wax moth. Those of an 11th group were injected with 0.01 ml of normal wax moth blood before being challenged. Three further groups of larvae were each injected with 0.01 ml of blood from the wax moth injected with Bacto nutrient broth (Difco), with 0.01 ml of normal rabbit serum, and with 0.01 ml of the antiserum of rabbits immunized with *P. aeruginosa* vaccine to check the specificity of passive immunity.

TABLE VII

Percentage mortalities in larvae of *G. mellonella* passively immunized against *P. aeruginosa*

Type of injection	No. of larvae in each group	Dose in bacteria per larva	Mortality, %*
Blood of wax moth vaccinated against *P. aeruginosa*			
2 hr after injection	60	280	60
4 hr after injection	60	280	47
12 hr after injection	60	280	37
20 hr after injection	60	259	30
24 hr after injection	50	161	28
48 hr after injection	60	105	60
72 hr after injection	60	252	95
Cells of vaccinated wax moth blood	50	100	70
Serum of vaccinated wax moth blood	50	100	68
Vaccinated wax moth blood heated to 60° C for 30 minutes	50	182	92
Normal wax moth blood	50	100	70
Blood of wax moth injected with Bacto nutrient broth (Difco)	50	100	80
Normal rabbit serum	60	99	55
Rabbit antiserum against P11-1 strain of *P. aeruginosa*	20	875	40
	70	112	32

*100% mortality occurred in equa lnumbers of control insects.

The results (Table VII) agree in general with those obtained by Zernoff with Danysz bacillus except it is probable that he used greater volumes of vaccine and of immunized blood to obtain such results. It is noteworthy that wax moth may be immunized with the antiserum of an immunized rabbit but cannot be immunized to an equal degree with normal rabbit serum or with normal wax moth blood and hardly at all with the blood of larvae injected with a foreign substance such as nutrient broth. This indicates that wax moth larvae evidently need a system containing antibody for passive immunization but, unlike mammals, they show no apparent reaction to the injection of a foreign serum.

Specificity of Immunization

Ishimori and Metalnikov (23) were among the early investigators who stated that insects could be immunized by nonspecific substances such as China ink, culture filtrates, broth, and heterologous vaccines. It is not reasonable to assume that, with such small groups of insects as they used, survival of two or three individuals resulted from protection by the nonspecific material, as these individuals might have had greater natural immunity to the infection. Ishimori and Metalnikov further stated that acquired immunity was manifested in 24 hours after injection of the immunizing substance and lasted during the life of the insect. In the present work, attempts were made to immunize *G. mellonella* larvae with heterologous substances, such as nutrient broth (Difco), physiological saline, and crystalline egg albumin but after a lethal dose of *P. aeruginosa* there was no greater survival in insects injected with these substances than in control insects given the same lethal dose.

To test the specificity of immunity in wax moth larvae after vaccination with 0.01 ml of the P11-1 strain of *P. aeruginosa*, vaccinated insects were challenged with three strains of *P. aeruginosa* of related antigenic properties, with the A.T.C.C. strain of *P. aeruginosa* 10145, and with two completely unrelated species, *Proteus* species isolated from grasshoppers and *Serratia marcescens* Bizio. Table VIII indicates that with the related strains of *P.*

TABLE VIII

Percentage mortalities in *G. mellonella* larvae from challenge doses of several strains of bacteria after vaccination with *P. aeruginosa*, strain P11-1, and after vaccination with a vaccine homologous to the challenge strain

Challenge strain	No. of larvae in group	Dose in bacteria per larva	Mortality, %		
			Vaccination with:		
			P11-1 vaccine	Homologous vaccine	Control
P. aeruginosa 46-4B	50	925	30	28	100
P. aeruginosa 284-1A	40	700	40	40	100
P. aeruginosa 344-1A	40	840	50	50	100
P. aeruginosa 10145	40	560	45	10	80
Proteus sp.	60	2100	80	8	100
S. marcescens	60	700	72	22	72

aeruginosa, and even with the antigenically distinct strain 10145, the same degree of protection was obtained as long as the vaccine was homologous to the bacterial species. However, very little protection was obtained for unrelated species. It may be concluded that immunization in larvae of *G. mellonella* is not necessarily strain-specific, as in mammals, but is possibly species-specific. The degree of protection against unrelated species of bacteria, such as *S. marcescens* or *Proteus* sp., is slight, if indeed it exists. The results suggest that acquired protection may be more frequently specific than nonspecific.

Immune Responses of Wax Moth Larvae to Other Bacterial Antigens

The following are preliminary findings on immune responses of wax moth larvae to two species of bacteria unrelated to *P. aeruginosa*.

S. marcescens strain CM4–1 (46) is pathogenic to the wax moth upon inoculation; the LD_{50} is about 40. It was not as easy to immunize wax moth larvae with a heat-killed (60° C for 30 minutes) vaccine of *S. marcescens* as with *P. aeruginosa* vaccine. Mortality in vaccinated groups sometimes reached a maximum of 60 to 70%. However, the increase in antigenicity for rabbits of *S. marcescens* in vaccinated wax moth blood was as great as, if not greater than, that observed with *P. aeruginosa*.

Bacillus cereus Frankland and Frankland, strain CM1-3 (47), is mildly pathogenic to wax moth larvae by injection; the LD_{50} is about 1500. So as not to introduce living material into the larvae, the vaccine was prepared by boiling a physiological saline suspension of *B. cereus* (rods and spores) for ½ hour. Only a low percentage of insects were protected by vaccination with 0.01 ml of this suspension. The blood of vaccinated larvae showed slight, if any, potentiation of the antigen for rabbits. These results may indicate that the antigen of *B. cereus* is not enhanced in vaccinated wax moth blood, though the possibility that the method of preparation of the vaccine probably had a harmful effect on the antigen must not be overlooked; it was known to be difficult to immunize rabbits with heat-killed cultures of *B. cereus* (47).

Immune Responses of Other Insects to *P. aeruginosa* Antigen

The wax moth was used as the experimental insect in most of the earlier investigations on insect immunity. It was used for most of the detailed work in this investigation because of the ease with which it can be reared and maintained in large numbers and because it has a relatively large blood volume. Preliminary experiments on responses of other insects to *P. aeruginosa* antigen are reported briefly to exclude the possibility that the immune response of the wax moth is vastly different from that of other species.

Silkworm Larvae

P. aeruginosa is less pathogenic for the silkworm, *Bombyx mori* (L.), by inoculation than for the wax moth; the LD_{50} for silkworm larvae is about 74

bacteria. It was possible to immunize silkworm larvae against *P. aeruginosa* by vaccination, though the difference in mortalities between immunized and control insects was not as pronounced as in the wax moth. Only 345 bacteria were used as a challenge dose for vaccinated and control silkworms, but 1200 bacteria were required to produce 100% mortality in the controls. It was impossible to duplicate the experiment to check further on the observed differences, because of limited numbers of test insects.

Duplicate tests on enhancement of antigenicity of *P. aeruginosa* in silkworm blood indicated that an 8- to 10-fold increase occurred, comparable to that described for the wax moth. No search was made for antibodies in the blood of the silkworm or for an inhibitory factor against *P. aeruginosa*.

Red-backed Cutworm Larvae

Larvae of the red-backed cutworm, *Euxoa ochrogaster* (Guen.), are more difficult to infect with *P. aeruginosa*; the LD_{50} by injection is about 124 bacteria. These larvae back-bleed freely, so that it is always difficult to be certain of the volume of culture or antigen that a larva retains. However, in two groups of 50 cutworm larvae challenged with 987 bacteria, there was 8% mortality in the vaccinated group and 74% mortality in the control. The antigenicity for rabbits of *P. aeruginosa* antigen in immune cutworm blood is about two to four times that of standard antigen; evidently the antigen is not enhanced to as great an extent by the blood of immune cutworm larvae as by the blood of immune wax moth or silkworm larvae.

Grasshoppers

Adults of the grasshopper *Melanoplus bivittatus* (Say) are very susceptible to injected doses of *P. aeruginosa*; the LD_{50} is 10 to 20 bacteria (5). Grasshoppers may be considered to be as susceptible as the wax moth, but are more difficult to immunize. Mortality in vaccinated groups was usually 50 to 60% as compared with 30% in the wax moth. Though the percentage volume of blood in the grasshopper was not determined and an accurate determination of antigen dilution was not possible, the antigenicity of *P. aeruginosa* for rabbits is apparently only very slightly enhanced in grasshoppers. Immune responses evidently differ between orders of insects.

General Discussion and Conclusions

The most notable differences in the observations on immune responses of insects reported herein as compared with those of other workers are: enhanced antigenicity for rabbits of some bacterial antigens in the blood of immune insects; isolation of the active antigen from immune insect blood by zone electrophoresis on starch; location of the enhanced antigenic component in blood serum rather than in cells. A significant difference in the method of this investigation from that of earlier investigations was the study of the antigen within the insect; previously most attention had been focused on a search for antibodies. Some other findings contrary to those of other workers are: the immunity is not as nonspecific as previously believed (8, 23, 60);

and it was not possible to protect wax moth larvae with nonspecific substances, such as broth, though it is possible to immunize them passively with the blood of rabbits immunized against the homologous antigen. The following observations are in agreement with those made by previous authors: it is possible to immunize lepidopterous insect species by vaccination; the immunity is of short duration (about three days) and may be conferred actively or passively; and it was not found possible to demonstrate true antibodies in immunized insect blood.

As wax moth blood apparently does not act as a true adjuvant and as lysis of the antigen within wax moth blood cannot be the chief factor responsible for enhancing the antigenicity, the antigen may be altered in some way and thereby made more efficient in its ability to produce antibodies.

There are references in the literature to parts of antigens being more antigenic than the whole. For example, Evans and Perkins (10) stated that an extract of pertussis vaccine induced early immunity as efficiently as the whole vaccine, and Goebel *et al.* (16, 17) stated that the activity of the antigenic fraction of *Shigella paradysenteriae* (Flexner) was increased during purification. Contrary to this, Topley *et al.* (50) reported that the purified antigen of *Bacterium typhosum* (=*Salmonella typhosa* (Zopf) White) was slightly less effective at immunizing than whole cells.

Though there is no definite evidence that the *P. aeruginosa* antigen is broken down, whole bacterial cells are probably not necessary. When the insect cells are separated from the serum by centrifugation it is assumed that the bacterial cells are also removed as none can be found in the wax moth blood serum; however, this serum is as antigenic as is the whole blood (Table III). It is rather remarkable that a strong agglutination reaction is obtained when no cells are present, and when the antigen may be in soluble form. The injection of *P. aeruginosa* and wax moth blood into a rabbit is actually a combined injection of two antigens, as the wax moth blood is also antigenic. Greenberg and Fleming (18) found that a combination of diphtheria toxoid with other antigens enhanced the immunizing efficiency of the diphtheria toxoid for guinea pigs. The efficiency was at least doubled with the addition of pertussis vaccine, and the addition of a third antigen, such as that of tetanus, increased the efficiency by at least 5 to 10 times. The immunizing efficiency of *P. aeruginosa* vaccine for rabbits is evidently increased to a similar degree when it is injected in immune wax moth blood.

Landy *et al.* (27) reported that when a somatic antigen of the "Boivin" type is freed of protein, the resultant lipopolysaccharide exhibits no loss in antigenic activity, but instead shows considerable increase. It is unknown whether wax moth blood which may be considered particulate in form serves as a carrier for a chemically changed *P. aeruginosa* antigen, but this possibility exists. Johnson *et al.* (24) discussed the ability of bacterial endotoxins to enhance antibody formation to a variety of protein antigens. They pointed out that such an investigation is a rather formidable problem, as so little is known of the mechanisms involved. When they injected *P. aeruginosa*

endotoxin into rabbits in conjunction with ovalbumin the antibody response to ovalbumin was greatly increased. The possibility of liberation of an endotoxin in wax moth blood working in conjunction with a soluble antigen may be considered.

The increase in antigenicity for rabbits of *P. aeruginosa* vaccine within wax moth blood is pronounced despite a large error in estimation (up to 100%). The estimated volumes of antigen may be high, for all estimations were based on the assumption that the injected antigen remains in the blood and that none is deposited in the tissues. Moreover, Beard (2) reported that the blood volume of insects is increased after the injection of certain foreign materials, such as sodium chloride, so that the antigen may be even more dilute than is reported herein.

The present work was concerned with immune responses to bacterial pathogens only. There are reasons to postulate that antigenic enhancement may be obtained with nonpathogens, but that only the antigens of Gram-negative bacteria are enhanced within the blood of immunized insects and that this phenomenon occurs most commonly in lepidopterous insects.

The observation on the enhanced antigenicity for rabbits of some bacterial antigens in the blood of vaccinated insects merits further consideration. In the general field of immunology any indication of a new means of increasing the efficiency of an antigen is important because many antigens are not as efficient as they might be and also have serious side effects. The action must evidently result from more than retarded adsorption and continuous antigenic stimulation caused by the presence of wax moth blood, as the material eluted by electrophoretic separation is not particulate (in the same sense as are true adjuvants) but the potentiation of the antibody response by it is still evident. The chemical aspect of this problem is clearly as important as the bacteriological and it seems likely that elucidation of the mechanism may come only through an immunochemical study.

Much work evidently remains to be done to characterize further this reaction indicating that the antigen is bound to a blood protein. It is not remarkable that Krieg (25) did not observe binding of *Staphylococcus aureus* antigen in the blood of several insects, as it is well known that great variation in results may occur from one electrophoretic technique to another. The possibility that the antigen in wax moth blood may be combined with a globulinlike component, as in mammals, should be investigated.

As any injected dose of *P. aeruginosa* in excess of 100 bacteria usually causes 100% mortality to wax moth larvae, it may be assumed that, under the conditions of testing, larvae of *G. mellonella* have little or no natural immunity to *P. aeruginosa*. As stated by previous investigators, immunity in these insects develops rapidly and is at a maximum by 24 hours. However, early investigators used such small groups of insects and such indeterminate doses that it was not possible to determine the degree and specificity of protection. Immunization of insects was generally attempted with old or otherwise attenuated cultures rather than with standardized vaccines, and

dosages of antigen and bacteria were referred to by size of drop or by volumes without supporting numbers, or occasionally (30) as strong or weak doses.

The concentration of vaccine (in proportion to size of insect) used to immunize the wax moth is undoubtedly greater than that used to immunize mammals. Active immunization of mammals, with some exceptions (3), does not necessarily last during the life of the individual. It may not be remarkable that immunity in insects is developed rapidly and that it does not persist for a long time.

In studies of mammalian immunity there have been a few reports of specific immunity not associated with antibody production. Evans and Perkins (10, 11) reported that a single intraperitoneal injection of pertussis vaccine in mice produced a substantial degree of immunity within a few hours to an intracerebral injection of *Hemophilus pertussis* Holland: no detectable antibodies were present when immunity first occurred or 3 days after, when it was still at a high level. They stated that two distinct immune responses were produced in mice in response to the injection. The first, of the interference type, is transient, occurs rapidly, reaches its maximum effect in about 10 days, and then quickly fades; the second, of an antibody type, occurs rapidly and reaches a high level in 20 to 40 days, when it shows no sign of waning. The possibility should not be overlooked that such an interference type of immunity, analogous to that known to occur in some virus diseases of mammals, exists in insects, as the rapid development of immunity and its transient nature closely parallel the interference type in mice, and perhaps only in rare cases does immunity of the antibody type exist in insects.

Paillot (38), in 1922, postulated that humoral reactions in insects were caused by chemical changes in the blood, which in turn changed or destroyed the bacteria. Terzian (49) suggested that host immunity of mosquitoes to malarial infection may depend upon the addition or depletion of specific physiological or metabolic factors that function in low concentration. Whereas Terzian referred to innate immunity in the mosquito, the present investigation suggests that an accumulation of a chemical component, perhaps of globulinlike nature, may function in the acquired immunity of insects. Further evidence suggesting the existence of a physicochemical change in the insect was the obesrvation that, though normal wax moth blood melanizes very quickly, no melanization occurred in hundreds of pooled samples removed from vaccinated individuals. (Grasshoppers, which show little increase in antigenicity of *P. aeruginosa* antigen for rabbits, do not exhibit this property.) Lerner and Fitzpatrick (28) suggested several possible causes for the inhibition of melanin formation, many of them chemical. Though the reason for this inhibition in the blood of immune wax moth is undetermined, it is apparently not due to an effect as simple as a change in pH for the reduction in pH of the blood of vaccinated larvae was very slight.

The data herein presented suggest that the immune responses of insects are more complex than was originally thought. More information on the immune responses of insects can probably be gained by more diverse methods, such as electrophoresis, than by a conventional search for antibodies.

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DEHYDROGENASE ACTIVITY IN SOILS[1]

I. L. STEVENSON

Abstract

The reduction of 2,3,5-triphenyltetrazolium chloride to triphenylformazan has been used to estimate the dehydrogenase activity of the soil microflora *in situ*. Preliminary investigations suggest that the formation of the formazan may be used as an index of endogenous respiration in soil since a significant correlation has been obtained between dehydrogenase activity and the oxygen uptake of a number of different untreated soils. A direct relationship also was observed between formazan formation and the respiration of a soil sampled at intervals after the application of fresh plant material. In a survey of a number of field soils no relationship was found between dehydrogenase activity and bacterial numbers; however, the two parameters were parallel during the decomposition of flax residues added to one of the soils.

Introduction

In the past few years a number of investigators have devoted considerable time to the development of enzymatic tests for determining microbial activity in soil. Most of these enzyme techniques involve the addition of known substrates to soil samples; activity is then estimated by determining the end products formed. Thus the action of saccharase, urease, asparaginase, protease, phytase, and amylase in soils have been determined by a variety of methods (1, 2, 3, 4, 5). A number of controversies have arisen out of these studies. Some authors have found a relationship between specific enzyme activity in soil and known indices of microbial activity (8, 9). However, in most instances no relationship could be found between these enzyme tests and total numbers of microorganisms, carbon dioxide production, nitrification, or ammonification in soils (4, 6). The failure of such tests to serve as reliable criteria of total microbial activity stems from the fact that most of the substrates used resulted in an adaptive response by the specific organisms or groups of organisms involved. Drobnik (2) has pointed out that, although enzyme tests are suitable for qualitative studies of various soil processes, they are not applicable for the quantitative estimation of total activity.

Lenhard (7) has recently used 2,3,5-triphenyltetrazolium chloride to study microbial activity in soil. The reduction of the tetrazolium salt to a stable colored formazan in soil was shown to be influenced by substrate concentration as well as soil type. No attempt was made to relate formazan formation to known criteria of microbial activity.

The present paper describes the application of a modification of Lenhard's technique and presents evidence for the reliability of the dehydrogenase test as a means of estimating total microbial activity in soils.

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Materials and Methods

Dehydrogenase Test

In Lenhard's original description of the method, soil samples were brought to 90% of their water-holding capacity and incubated for 24 hours prior to the addition of 2,3,5-triphenyltetrazolium chloride (TTC). The samples were then reincubated for 24 hours and extracted with 50 ml volumes of methanol to remove the red-colored triphenylformazan (TPF). In preliminary studies in this laboratory it was found that the initial incubation in the absence of TTC was unneccessary in that identical results were obtained if TTC was added directly and extracted at the end of 24 hours' incubation. Prolonged incubation of soils plus TTC indicated that TPF was produced in soils at a relatively constant rate during the first 48-hour period, after which the rates of formazan formation decreased rapidly. In this respect the amount of TPF or its hydrogen equivalent appeared as an accurate estimate of activity. Further studies showed that in many soils 50 ml of methanol was insufficient to extract completely the TPF from soil. A practice was made of passing methanol through the soils until the extractant appeared colorless. The amounts used were recorded for individual soils and appropriate corrections made in the colorimetric determination of TPF.

In these studies the dehydrogenase test was carried out as follows: 20 g of fresh soil was mixed in a 50-ml beaker with 200 mg of dry $CaCO_3$ and brought to 90% water-holding capacity with water containing 2.0 ml of a 1% solution of TTC. The soil was then thoroughly mixed and the surface tamped to prevent access of air. The sample was then incubated at 30° C in a humidified incubator (R.H., 70%) for 24 hours. Following incubation, a 25-ml volume of methanol was added to the beaker and stirred for 5 minutes. The resulting slurry was washed into a Büchner filter (Whatman No. 5 paper) and extracted with successive aliquots of methanol. Volumes of extractant used for individual samples were then recorded.

The density of colored extract was determined spectrophotometrically at a wave length of 485 mμ, using methanol as the reference blank. Concentrations were calculated by comparison with a standard curve of TPF in methanol. Results were recorded in volumes of hydrogen transferred during the reduction of TTC to TPF in 20 g soil according to the equation:

2,3,5-triphenyltetrazolium chloride + 2H$\rightarrow$triphenylformazan + HCl.

The formation of 1 mg of TPF requires 150.35 μl H.

Soil Respiration Studies

Oxygen uptake of soils was determined by Warburg techniques previously described (10). The accumulated O_2 uptake of 4.0 g soil in 6 hours has been taken as an estimate of the respiratory activity in the soils studied.

Soils

The soils used were selected from various sites in Carleton County, Ontario. Those designated as Us, Ms, K, X, and NG have been previously described

(10), while North Gower clay (NGc), Rideau clay (Rc), Grenville loam (Gl) are representative of arable soils in the district. Soils N, X, Y, and Z were from the fertility plots of the Central Experimental Farm.

All the soils were held in a moist condition as they arrived from the field. Suitable corrections for variations in moisture were applied in carrying out the dehydrogenase test.

Results

Studies were undertaken to compare dehydrogenase activity as measured by the reduction of TTC and the respiratory activity (oxygen uptake) of a number of different soils. The results of these studies along with the bacterial numbers in the samples are presented in Table I. It will be noted that an association exists between oxygen uptake and dehydrogenase activity in the soil samples. This is illustrated graphically in Fig. 1, where a significant correlation is shown between the two parameters. No relationship was apparent between bacterial numbers and either oxygen uptake or TPF formation. In view of the relationship observed between enzyme activity as measured by the dehydrogenase test and oxygen uptake in the different soils further tests were conducted to determine whether the methods would agree within one soil as microbial activity fluctuates due to the addition of organic material. Samples of a relatively non-fertile soil (soil G, Table I) were supplemented by fresh crop residues of flax, alfalfa, wheat, timothy, and red clover. The treated soils were held under field conditions, sampled at intervals to 35 days, and tested for dehydrogenase and respiratory activity. Plate counts for bacterial numbers were also made. The relationship found between oxygen uptake and dehydrogenase activity is presented in Fig. 2. Again a significant correlation occurs, indicating that as microbial activity of the soil fluctuates due to the decomposition of the organic material, corresponding changes occur in dehydrogenase activity and oxygen uptake.

TABLE I

Comparison of dehydrogenase activity, O_2 uptake, and bacterial counts in different soils

Soil	Index of activity		
	O_2 uptake (X)	Dehydrog. (Y)	Bacteria counts
X	96*	46†	570‡
Y	84	51	540
G	51	54	75
Z	91	66	390
Rc	115	71	300
NGc	51	91	490
N	129	92	140
Kg	269	93	81
Ms	130	94	570
Gl	306	227	290
Us	337	308	280
NG	438	415	390

*μl O_2 per 4.0 g soil (6 hr totals).
†μl H per 20.0 g soil (24 hr).
‡Bacteria counts $\times 10^6$ per 1.0 g.

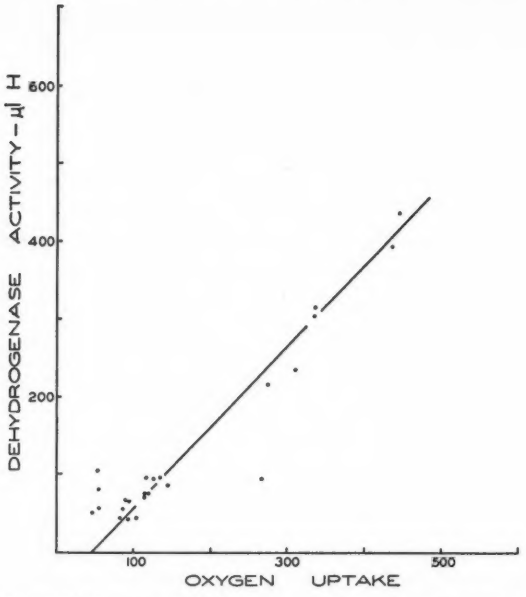

FIG. 1. Relationship between dehydrogenase activity and oxygen uptake of a number of fresh soils (dehydrogenase, µl H per 20.0 g soil (24 hr); oxygen, µl O_2 per 4.0 g soil (6 hr)). $r = .837$, $N = 24$.

The changes observed in bacterial numbers and dehydrogenase activity during the decomposition of the flax residues in soil G are presented in Fig. 3. Control soil data are also presented to illustrate the normal fluctuations in activity in the untreated soil. In general, dehydrogenase activity paralleled bacterial numbers in both the control and supplemented soil. A discrepancy may be noted during the first three days of decomposition of the flax residue when an immediate increase in enzyme activity occurred despite little or no increase in bacterial numbers. It is reasonable to assume that during the initial stages of decomposition the soil flora becomes metabolically more active prior to multiplication of the organisms.

In previous unpublished studies in this laboratory it had been noticed that leaching a fresh soil increased the respiratory activity of the sample (oxygen uptake), presumably through disaggregation and stimulation of the organisms. Similar studies were undertaken to determine the effect of leaching on dehydrogenase activity in fresh soil samples. Samples, consisting of 200 g of soil, were leached by three successive Büchner filtrations with a total of 200 ml distilled water. The extracted samples were then tested for dehydrogenase activity and oxygen uptake in the usual manner. Suitable moisture corrections were applied. Samples of the extract were also (*a*) concentrated on a rotary evaporator or (*b*) ashed in a muffle furnace. These concentrated or ashed extracts were then added to the leached soils in the original concentration (i.e. 1.0 g leached soil received the concentrated or ashed extract from

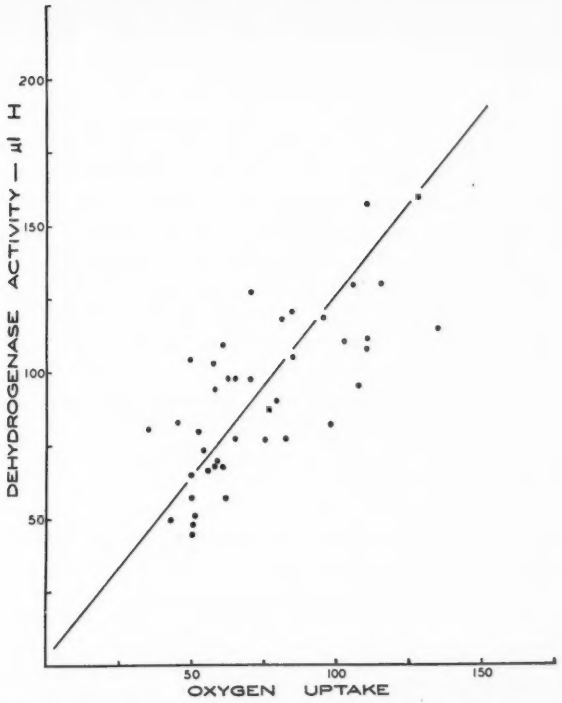

FIG. 2. Relationship between dehydrogenase activity and oxygen uptake during the decomposition of plant residues in soil (dehydrogenase, µl H per 20.0 g soil (24 hr); oxygen, µl O_2 per 4.0 g soil (6 hr)). $r = .511$, $N = 43$.

1.0 g soil). Data from these experiments are presented in Table II. In both the soil samples studied leaching of the soil markedly increased both respiration and dehydrogenase activity. Return of the concentrated extracts to the leached samples failed to affect oxygen uptake (which remained high) but lowered dehydrogenase activity to approximately the level of the unleached samples; on the other hand, incorporation of ashed extracts failed to affect dehydrogenase activity.

TABLE II

Effect of leaching on dehydrogenase activity and O_2 uptake in soil NG

Treatment	Soil A*		Soil B*	
	Dehydrog.	O_2 uptake	Dehydrog.	O_2 uptake
Control soil	407†	356‡	336†	197‡
Leached soil	766	601	608	324
Leached soil + extract	487	614	356	310
Leached soil + ashed extract	—	—	621	328

*Two samplings of soil NG.
†µl H per 20.0 g soil (24 hr).
‡µl O_2 per 4.0 g soil (6 hr totals).

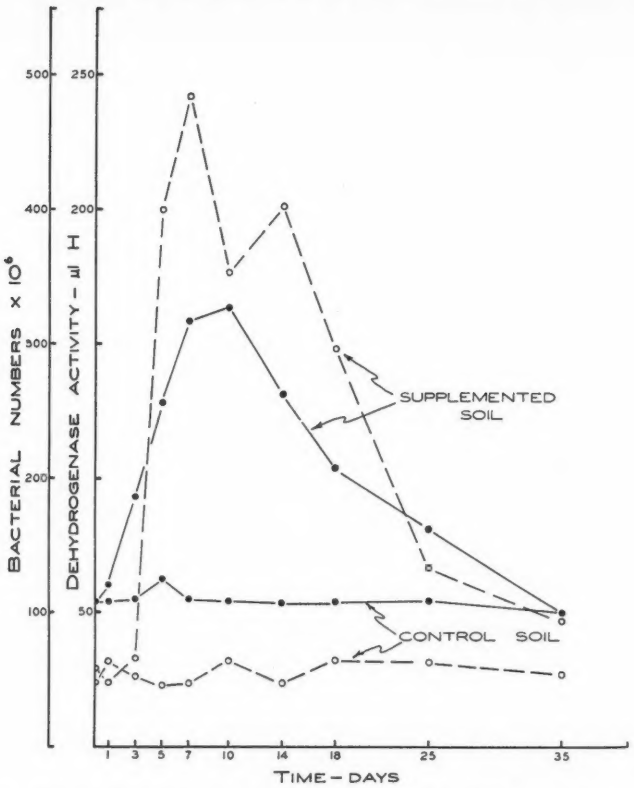

FIG. 3. Relationship between dehydrogenase activity (solid lines) and numbers of bacteria (broken lines) during decomposition of flax residues in soil (dehydrogenase activity, µl H per 20.0 g soil (24 hr); oxygen, µl O_2 per 4.0 g soil (6 hr)).

The action of the extracts suggests the presence of some inhibitory or antagonistic factor which interferes with TPF formation in soil, either through inhibition of respiratory enzymes in the soil or possibly by providing a more suitable hydrogen acceptor than TTC.

Discussion

The use of 2,3,5-triphenyltetrazolium chloride as an indicator of enzyme activity in soils has been investigated. In the absence of oxygen the tetrazolium salt acts as the terminal hydrogen acceptor for dehydrogenase systems and a colored formazan is produced. In soil the reduction of the indicator is dependent solely on the normal enzymatic activity of the cells under the anaerobic conditions of the test. In this respect the method proves advantageous over previously described enzyme tests for soil in that no additional substrate is required, thus precluding the preferential stimulation of any group or groups of organisms.

In these studies attempts have been made to relate dehydrogenase activity to such known criteria of microbiological activity as oxygen uptake and bacterial numbers. A direct correlation has been obtained between the reduction of TTC and the respiratory activity (oxygen uptake), both in different soils and in one soil during the course of decomposition of organic supplements. A similar relationship was also obtained between dehydrogenase activity and bacterial numbers in the decomposition studies. In view of these results it would appear that the colorimetric determination of TPF formed also provides a reliable index of microbial activity in soil.

Some data have also been presented indicating that the reduction of TTC can be increased through leaching of the soil. Although a similar effect is noted with oxygen uptake of leached samples, differences are noted when the extracts are returned to the samples. Replacement of the concentrated extracts does not lower the oxygen uptake of soils but it does lower dehydrogenase activity to approximately its original level. It is felt that leaching has stimulated the oxidative capacity of the soils through disaggregation of organisms and possible solubilization of nutrients, processes which are not reversed on replacement of the extracts. In the case of dehydrogenase activity leaching has apparently removed an inhibitor which can re-exert its effect on being returned to the soil. The possibilities exist that either a direct inhibition of the dehydrogenase system occurs or organic substrates are present which exert an antagonistic effect through competition for available coenzyme and related substances. These effects have been noted in all soils investigated and further studies are being conducted along these lines.

Acknowledgment

The author wishes to express his appreciation for the technical assistance of Mrs. Kristine Orlik-Rückemann.

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VARIABLES INFLUENCING THE PREPARATION OF INFLUENZA VACCINES WITH FORMALDEHYDE[1]

JOHN R. POLLEY

Abstract

The effect of temperature, time of treatment, and the pH of the medium on the preparation of influenza vaccines with formaldehyde has been studied. It was found that both the infectivity and the hemagglutinin were destroyed more quickly as the pH was increased above pH 6 and the margin of safety that exists between virus inactivation and loss of antigenicity increased as the pH was decreased to about 6. From the experimental data it was possible to select numerous conditions of treatment which had a large margin of safety between virus inactivation and loss of antigenicity. Vaccines prepared with formaldehyde under various conditions and then lyophilized had retained their antigenicity after storage for 2½ years.

Introduction

Early work on the preparation of influenza vaccines involved treatment of the infected allantoic fluid or virus suspension with formaldehyde in the cold. A wide range of formaldehyde concentrations and exposure times were used. Hare (1) used formaldehyde at a concentration of about 0.01% in the cold for 4 days. Treatment with 0.4% formaldehyde at 4° C was used by Hirst (2). In 1945 Salk (6) described a process employing a concentration of 0.05% at 4° C for 48 hours. Later it was shown in this laboratory that the pH of the suspending medium of the virus was an important factor governing the stability of the hemagglutinin to formaldehyde treatment and a temperature of 45° C was selected for the preparation of non-infective viral antigens (4). It was found that the suspension of the influenza virus in isotonic phosphate buffer at pH 6 afforded maximum stability for the hemagglutinating and complement-fixing components of the virus to inactivation with formaldehyde.

It is apparent from the above that a wide range of reaction conditions can be used for the destruction of the infectivity of influenza virus suspensions. Although the hemagglutinating and complement-fixing components are relatively stable under appropriate conditions to rather vigorous treatment with formaldehyde, it does not follow that the resultant non-infective virus suspension produces a good antibody response or protection to challenge with live virus. Since it is still not known what combination of pH and temperature with formaldehyde gives the best vaccine, an investigation of the optimal conditions for the preparation of influenza vaccines with formaldehyde was carried out.

[1]Manuscript received January 10, 1959.
Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

Observations

For a quantitative study of the effect of formaldehyde concentration, temperature, time of treatment, and pH on the rate of destruction of the antigenicity and the infectivity of influenza virus, suspensions of this virus were prepared by centrifuging the allantoic fluid specifically infected with influenza A (PR8) at 19,000 *g* for 1 hour and resuspending the virus in isotonic phosphate buffers at various pH values. The suspensions were adjusted to the same initial hemagglutination titer. Formaldehyde in the form of a 10% aqueous solution was added to the suspensions to give a concentration of 0.01% in each. The treated samples and untreated controls were placed in a water bath at 37° C. After various time intervals, portions were removed and tested for hemagglutination titer and infectivity, as described previously

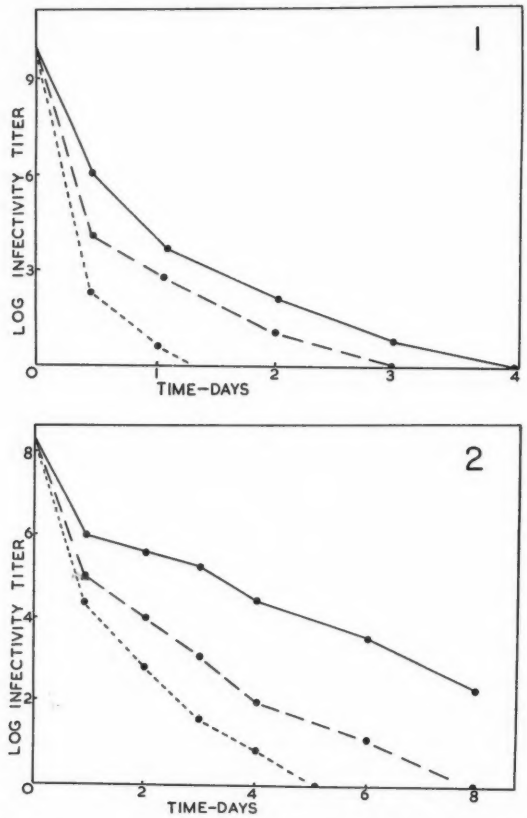

FIG. 1. Effect of pH on the rate of inactivation with 0.01% formaldehyde at 37° C. ●———● pH 6, ●— — —● pH 7, ●- - - -● pH 8.5.

FIG. 2. Effect of pH on the rate of inactivation with 0.01% formaldehyde at 4° C. ●———● pH 6, ●— — —● pH 7, ●- - - -● pH 8.5.

(4). The effect of the pH of the suspending medium on the rate of inactivation is shown in Fig. 1, from which it is evident that the infectivity was destroyed more rapidly as the pH was increased. The samples treated at pH 6 and 7 had retained their hemagglutination titers even after 8 weeks under these conditions whereas the sample treated at pH 8.5 began to show a falling titer after 4 days.

The experiment above was repeated at a temperature of 4° C. The results are shown in Fig. 2. In this case, the effect of pH appeared to be more pronounced than at 37° C. The sample treated at pH 7 became non-infective after 8 days while that treated at pH 6 required 14 days for inactivation. The hemagglutination titers of these suspensions showed no significant change after 12 weeks.

Similar experiments were conducted using 0.1% and 0.25% formaldehyde. Again it was found that with these higher formaldehyde concentrations, increasing the pH of the medium caused not only a more rapid loss of infectivity but also a greater destruction of the hemagglutinin content. The results are summarized in Table I and illustrate for each method the difference in the time required for virus inactivation and the time it takes for the destruction of the antigenicity, as indicated by the loss of hemagglutinin. By relating the inactivation curves to the corresponding hemagglutination titers it was possible to select a number of reaction conditions which permitted virus inactivation without significant loss of hemagglutination titer. In a previous study using beta-propiolactone (5), the relationship between the hemagglutination titer of a virus vaccine and its antigenicity was demonstrated: those virus suspensions, which after inactivation were found to have retained most of their hemagglutination titer, were also shown to produce a good antibody response in guinea pigs.

TABLE I

Effect of formaldehyde treatment under various conditions on the infectivity and hemagglutinin of influenza A virus (PR8)

HCHO, %	Temp., °C	pH	Time for inactivation, days	Stability of hemagglutinin, days
0.01	37	8.5	1 (1–2)	4
0.01	37	7	3 (2–5)	>56
0.01	37	6	6 (4–8)	>56
0.01	4	8.5	4 (4–8)	28
0.01	4	7	8 (6–10)	>100
0.01	4	6	14 (10–16)	>100
0.1	37	8.5	½	½
0.1	37	7	1 (½–2)	2
0.1	37	6	2 (1–3)	6
0.1	4	8.5	2 (1–3)	7
0.1	4	7	4 (3–6)	>60
0.1	4	6	6 (5–8)	>100
0.25	4	8.5	½	7
0.25	4	7	1 (1–2)	>30
0.25	4	6	2 (1–3)	>50

It remained to determine the optimal conditions of formaldehyde treatment for antibody response and immunizing capacity. A concentrated suspension of influenza A (PR8) virus was prepared by centrifuging the allantoic fluid as before and resuspending the virus in about 1/30 the previous volume of saline. This concentrated suspension was dialyzed for 24 hours at 4° C against saline. Portions of it were then diluted about 1/10 with isotonic phosphate buffers of various pH values, so that all final virus suspensions prepared from the same common pool of virus had the same hemagglutination titers of 1:5120. Formaldehyde was added as before to each suspension to give the desired concentration. The suspensions were placed in water baths at the designated temperatures. The exact reaction conditions used for the preparation of each vaccine are shown in Table II. At the completion of treatment, the excess formaldehyde was neutralized by the addition of 30% (w/w) dibasic ammonium phosphate solution (0.25 cc per 10 cc of 0.1% formaldehyde-treated vaccine) and the vaccines were lyophilized as described previously (4). The dried vaccines were reconstituted to their original volume with distilled water and were tested for infectivity and hemagglutination titer. The vaccines were inoculated into 10 young adult guinea pigs (about 300 g) per group, each animal receiving 0.5 cc by the intraperitoneal route. Samples of blood were obtained after various time intervals from 6 animals selected at random. The sera were tested individually for specific antibody by the hemagglutination-inhibition technique. The results are shown in Table II.

The experiment described above demonstrated that influenza vaccines prepared with formaldehyde under very different experimental conditions induced specific antibody response in guinea pigs. It remained to be demonstrated that such vaccines would confer protection against challenge with live virus.

For this purpose, an influenza A virus, Shope's swine influenza strain adapted to mice, was used for the protection tests (5). Suspensions of this virus having the same hemagglutination titers were prepared in isotonic phosphate buffers of various pH values as described above. Portions of

TABLE II

Comparison of influenza A (PR8) vaccines prepared with formaldehyde under various conditions as measured by antibody response in guinea pigs

Temp., °C	pH	Time, days	Concn., %	Titer after treatment	Titer after lyophilization	Infect.	Antibody level after 1 month	Antibody level after 6 months	Antibody level after 1 year
37	6	7	0.01	10,240	2,560	+	—	—	—
37	7	4	0.01	10,240	5,120	−	6,449*	907	837
37	8.5	2	0.01	10,240	10,240	−	7,238	538	530
37	6	1	0.1	5,120	5,120	−	6,400	1,076	530
4	8.5	3	0.1	10,240	10,240	−	7,238	1,810	907
45	6	½	0.1	10,240	5,120	−	4,067	538	452
37	6	1	0.25	5,120	5,120	−	3,226	425	452
4	8.5	2	0.25	10,240	5,120	−	5,121	1,076	837
4	7	7	0.25	10,240	5,120	−	8,125	1,388	1,016

*Geometric mean of results with six individual sera.

TABLE III

Antibody response and protective effect in mice of influenza A (Shope swine influenza strain) vaccines prepared with formaldehyde

Conditions of treatment					Challenged after 1 month		Challenged after 6 months	
HCHO concn., %	Temp., °C	pH	Time, days	Resultant vaccine titer	Antibody titer	Survivors	Antibody titer	Survivors
0.01	37	7	4	5120	1280	30/30	320	33/40
0.01	37	8.5	2	5120	2560	28/30	320	39/40
0.1	4	8.5	3	2560	1280	30/30	640	39/40
0.1	45	6	½	2560	2560	29/30	640	38/40
0.1	37	6	1	1280	1280	28/30	320	37/40
0.25	37	6	1	2560	2560	29/30	640	35/40
0.25	4	8.5	2	5120	2560	28/30	320	36/40
				Controls	80	4/30	40	11/40

these suspensions were treated with formaldehyde under the conditions shown in Table III. The treated vaccines were then lyophilized, reconstituted to original volume, and tested for infectivity and hemagglutination titer. Each reconstituted vaccine was inoculated into a group of 90 mice, each mouse receiving 0.5 cc by the intraperitoneal route. After 30 days, 10 mice were selected at random from each group and the antibody level of their pooled sera was determined in the hemagglutination-inhibition test. Thirty mice from each group were then challenged by the intranasal route with a 1/10 dilution of the challenge inoculum, as described previously (5). After an additional 5 months, another determination of the antibody level was made as described above and the remaining 40 mice were challenged as before. The results are shown in Table III.

The relationship between the hemagglutination titer of the vaccine and the resultant antibody level and degree of protection was studied further in another experiment. An influenza A (Shope's swine virus strain) vaccine was prepared by treating the virus suspension with 0.1% formaldehyde at 45° C at pH 6 for 12 hours and then lyophilizing as before. The vaccine had a hemagglutination titer of 1:2560. Serial 4-fold dilutions of this vaccine were made with sterile saline and the four diluted vaccines together with the undiluted vaccine were each inoculated into groups of 80 mice, each mouse receiving 0.5 cc by the intraperitoneal route as before. After 1 month, a determination of the antibody level was made and 30 mice of each group were challenged as in the previous experiment. After another 5 months the antibody level was measured again and the remaining 30 mice were challenged. The results are shown in Table IV.

A number of these experimental vaccines were investigated for their stability on storage. Three vaccines which had been treated with formaldehyde and lyophilized 2½ years previously were tested. These vaccines had been stored in their glass ampoules at 4° C. Vaccine No. 1 had been treated with 0.1% formaldehyde at pH 8.5 for 3 days at 4° C; vaccine No. 2 with 0.1% at pH 6 at 45° C for 12 hours; and vaccine No. 3 with 0.25% at pH 6 at 37° C for 1 day. The dried vaccines were reconstituted to their original volume with distilled water. Forty mice were inoculated with each vaccine, each mouse

TABLE IV

Effect of influenza A (Shope swine influenza virus strain) vaccine titer on antibody response and protective effect in mice

	Challenged after 1 month		Challenged after 6 months	
Vaccine titer*	Antibody level	Survivors	Antibody level	Survivors
2560	640	36/40	320	38/40
640	320	38/40	160	34/40
160	320	38/40	160	34/40
40	160	26/40	80	25/40
10	20	9/40	40	20/40
Controls	20	5/40	20	12/40

*Expressed as hemagglutinin units per ml.

TABLE V

Antibody response and protective effect in mice of three influenza A lyophilized vaccines stored 2½ years

		Challenged after 1 month	
Vaccine No.	Titer	Antibody level	Survivors
1	2560	320	28/30
2	2560	640	30/30
3	2560	640	29/30
	Controls	40	8/30

receiving 0.5 cc intraperitoneally. After 1 month the antibody level of 10 mice was determined and the remaining mice were challenged with live virus as described in the experiments above. The results are shown in Table V.

Discussion

From Fig. 1 it can be seen that the rate of inactivation of the influenza A (PR8) virus is influenced by the pH of the medium, each increment of pH doubling approximately the rate of inactivation. A comparison of Figs. 1 and 2 shows that the effect of pH of the medium is even more marked at 4° than at 37° C. When the temperature was increased from 4° to 37° C, the rate of inactivation at pH 7 and 8.5 was approximately doubled whereas at pH 6 the rate was increased roughly five times.

When concentrations of 0.1% and 0.25% formaldehyde were used instead of the 0.01% above, the effect of the pH was still apparent but the over-all increased rate of inactivation due to the higher concentrations of formaldehyde made the effect of the pH less pronounced. As would be expected, an increase in the temperature of the reaction or in the formaldehyde concentration increases the rate of virus inactivation. However, there is also an increase in the rate of destruction of the hemagglutinin. Since the hemagglutinin appears to be closely associated with the immunizing capacity of the virus, it is necessary to consider the effect of inactivating agents not only on the rate of virus inactivation but also on the stability of the hemagglutinin. For this purpose, the results obtained by treatments with formaldehyde

under various reaction conditions are summarized in Table I to show both the time required for inactivation and the period of time for which the hemagglutinin was stable under these conditions. These results are more significant in vaccine preparation than the simple presentation of inactivation curves, which do not show the corresponding effect on antigenicity. For example, in Table I the rate of inactivation by treatment with 0.1% formaldehyde at 37° C at a pH of 8.5 is a straight line from zero time to complete inactivation in 8 hours. This graph has little significance, however, when it is noted from Table I that the hemagglutinin is destroyed equally rapidly.

The range of times of treatment with formaldehyde found for virus inactivation in replicate experiments is also shown in Table I. It will be noted that in some cases there is a considerable variation in the results found, a fact that is well known in the production of vaccines. This is especially true under conditions that barely produce inactivation. Without some measure of the corresponding antigenicity, it would be necessary to select inactivation times arbitrarily and to assess the resulting vaccines in animal experiments. From the data in Table I it is possible to select conditions in which there is a large interval or margin of safety between the time required for virus inactivation and the time at which the hemagglutinin begins to disappear. It is apparent that both the infectivity and the hemagglutinin are destroyed more quickly as the pH is increased above pH 6 and the margin of safety that exists between virus inactivation and loss of antigenicity increases as the pH is decreased to about 6.

In Table II are shown the results when nine vaccines were prepared using various conditions of treatment. In one case, treatment with 0.01% formaldehyde at 37° C at pH 6 failed to produce a non-infective product when used on a large scale. The other conditions of treatment have been found to be satisfactory on repeated tests. It can be seen that all vaccines came through the various methods of treatment and the lyophilization with little loss of hemagglutination titer. The antibody levels produced show variations but there is no significant difference either in the levels produced or in their persistence to indicate that one method of treatment was superior to another. It appears that vaccines with similar hemagglutination titers give comparable antibody responses on inoculation into experimental animals.

In Table III is shown the antibody response and protective effect in mice of seven different influenza vaccines prepared by various methods of treatment. Again, all vaccines were prepared with formaldehyde and lyophilized successfully. As in the experiment above, the antibody responses were so similar that they gave no indication that one method of treatment was better than another. It is apparent that all vaccines afforded a high degree of protection to challenge with live virus, even 6 months after a single dose.

When a vaccine was serially diluted in 4-fold increments prior to inoculation, it is evident from Table IV that there was also a corresponding decrease in the antibody response produced, as might be expected. When the mice

were challenged with live virus after 1 month, the degree of protection afforded is statistically significant at the 1% level down to a vaccine having a hemagglutination titer of 1:40. After 6 months, the antibody levels still show the graded response and the number of survivors with the 1:40 vaccine is just significant at the 1% level, using the tables of Mainland *et al.* (3). Thus, an influenza vaccine having a hemagglutination titer of 1:40 and producing an antibody level of at least 1:80 as measured in the hemagglutination-inhibition test has given significant protection in this experiment.

Of considerable importance is the fact that three vaccines prepared by different methods of treatment and lyophilization and then stored for $2\frac{1}{2}$ years have retained their hemagglutination titer, their ability to produce an antibody response, and their ability to immunize experimental animals. The presence of a significant hemagglutination titer on reconstitution (1:80 or greater) in these animal experiments gives a good indication that the vaccine will be a satisfactory immunizing agent.

These experiments have shown that influenza vaccines can be prepared with formaldehyde under a wide range of reaction conditions. Consequently, it is possible to select conditions of treatment which have a large margin of safety between virus inactivation and loss of antigenicity without having to resort to the use of concentrations of formaldehyde which are more marginal for safe inactivation. Under such optimal conditions the question of inactivation of the virus as a first-order reaction becomes of less importance.

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NOTES TO CONTRIBUTORS

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MANUSCRIPTS

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